Ticks and Tick-borne Diseases 10 (2019) 101279

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

Transient transfection of *Babesia ovis* using heterologous promoters

Catarina Rosa[a,b,*], Masahito Asada[c], Hassan Hakimi[c], Ana Domingos[b,d], Madalena Pimentel[a], Sandra Antunes[b,d]

[a] Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, 1649-003, Lisboa, Portugal
[b] Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (IHMT-UNL), R. da Junqueira 100, 1349-008, Lisboa, Portugal
[c] Department of Protozoology, Institute of Tropical Medicine (NEKKEN), Nagasaki University, Sakamoto 1-12-4, Nagasaki, 852-8523, Japan
[d] Global Health and Tropical Medicine, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (GHTM-IHMT-UNL), Rua da Junqueira, 100, 1349-008, Portugal

ARTICLE INFO

Keywords:
Babesia ovis
Transient transfection
Elongation factor-1alpha
Heterologous promoter
Luciferase

ABSTRACT

Babesia species, etiological agents of babesiosis, a recognized emerging tick-borne disease, are a significant animal and human health concern with a worldwide socio-economic impact. The development of genetic manipulation techniques, such as transfection technology, is pivotal to improve knowledge regarding the biology of these poorly studied parasites towards better disease control strategies. For *Babesia ovis,* responsible for ovine babesiosis, a tick-borne disease of small ruminants, these tools are not yet available. The present study was based on the existence of interchangeable cross-species functional promoters between *Babesia* species. Herein, we describe for the first time *B. ovis* transient transfection using two heterologous promoters, the *ef-1α-B* intergenic regions from *B. bovis* and *B. ovata*. Their ability to drive expression of a reporter luciferase in *B. ovis* supports their cross-species functionality. Also, the *ef-1α-B* promoter region from *B. ovata* resulted in statistically significantly higher luminescence values in comparison to the control, thus a possibly suitable promoter for stable gene expression. Evaluation of transfection efficiency using qPCR demonstrated that higher luminescence levels were due to promoter strength rather than a higher transfection efficiency. These findings represent a step forward in the development of methods for *B. ovis* genetic manipulation, an undoubtedly necessary tool to study this parasite basic biology, including its life cycle, the parasite interactions with host cells and virulence factors.

1. Introduction

The genus *Babesia* (phylum Apicomplexa, order Piroplasmorida) comprises tick-transmitted protozoan hemoparasites responsible for babesiosis. *Babesia ovis* is the main etiological agent of ovine babesiosis, a disease of small ruminants mainly transmitted by the tick vector *Rhipicephalus bursa* (Erster et al., 2016a, 2016b; Ranjbar-Bahadori et al., 2012; Shayan et al., 2007). Ovine babesiosis is acknowledged for having an important socio-economic impact, particularly in underdeveloped countries related with animal mortality, costs of the livestock treatment and productivity losses (decrease of milk, meat and wool production) (Aouadi et al., 2017; Horta et al., 2014). Also, the current treatment with the babesiacide imidocarb dipropionate causes food insecurity (milk contamination) and the drug is potentially carcinogenic (Belloli et al., 2006; McHardy et al., 1986; Santos et al., 2012). The use of chemical acaricides to target the tick vector conveys similar safety issues and its reliability is insufficient due to rise of acaricide resistance among *R. bursa* species (Enayati et al., 2010). The need for improved disease control strategies is clear and the application of a vaccination protocol has been recommended (Ekici et al., 2012).

Development of genetic manipulation methods for *B. ovis* can provide an essential breakthrough for the study of this parasite basic biology, including its life cycle dynamics, parasite-vector and parasite-host interactions, ultimately enabling rational design of more effective drugs and vaccines towards disease control (Antunes et al., 2017; Suarez et al., 2017). Despite advances in the maintenance of *in vitro* *Babesia* spp. cultures, including for *B. ovis* Israeli strain, availability of complete and well annotated genomes and practical tools for genetic manipulation remain a necessity to tackle novel research avenues (Antunes et al., 2017; Suarez et al., 2017). Transfection technology (incorporation and expression of foreign DNA or RNA) has been applied to several apicomplexan parasites for the study of promoter function and gene regulation, gene function and even for the development of a genetically modified parasite line to use as a vaccine delivery system (Oldiges et al., 2016; Suarez et al., 2017). Transient transfection systems that provide short-term expression of a reporter gene are essential

---

[*] Corresponding author at: Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (IHMT-UNL), R. da Junqueira 100, 1349-008, Lisboa, Portugal.
E-mail address: catarina.msilvarosa@gmail.com (C. Rosa).

https://doi.org/10.1016/j.ttbdis.2019.101279
Received 5 April 2019; Received in revised form 13 August 2019; Accepted 23 August 2019

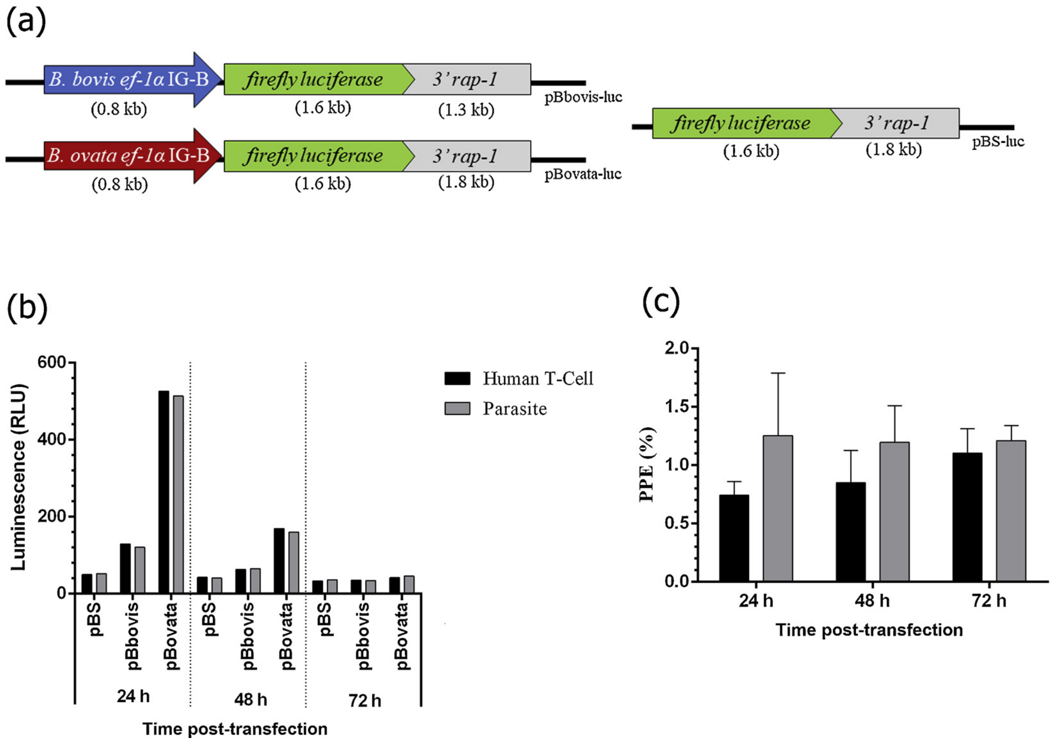

Fig. 1. Schematic diagram of the plasmids used for transient transfection and comparison between transfection with Human T-Cell and Parasite nucleofector solution. (A) Transient transfection constructs with *ef-1α-B* promoter sequence from *B. bovis* and *B. ovata* driving expression of luciferase as a reporter were developed as well as a promoter-less plasmid to use as negative control. (B) Luciferase expression in *B. ovis* transfected with plasmids pBbovis-luc, pBovata-luc and pBS-luc (negative control) using two different Amaxa nucleofector buffers: Human T-cell and Parasite. The luciferase assays were performed 24, 48 and 72 h post-transfection. RLU: Relative Luminescence Units. Data is expressed as the mean of the obtained RLUs for each transfection ± SD ($n = 1$). (C) Percentage of parasitized erythrocytes (PPE) in *B. ovis* cultures obtained 24, 48 and 72 h after nucleofection with the T-cell buffer and the Parasite buffer. Data is expressed as the mean PPE after three independent transfections ± SD ($n = 3$). Data obtained was compared using an unpaired two sample *t* test and no statistically significant difference was found among means ($P < 0.05$).

to define appropriate electroporation parameters and to find suitable promoters and termination regions to mediate transgene expression, being the first step to proceed to plasmid-based experimental systems that integrate the genome (Suarez et al., 2017; Suarez and McElwain, 2010). For *Babesia* spp., transfection techniques only emerged in the last decade after availability of the *B. bovis* genome sequence with several reports of transient and stable transfections systems (Suarez et al., 2004, 2006; Suarez and McElwain, 2008; Asada et al., 2012, 2015; Silva et al., 2016, 2018). Similarly, transient transfection followed by stable transfection has been performed in *B. ovata, B. bigemina* and *B. gibsoni* (Hakimi et al., 2016; Liu et al., 2018, 2017b, Silva et al., 2018, 2016)

The *elongation factor-1alpha* Intergenic Region (*ef-1α* IG) contains strong promoters since the Ef-1α protein is a key component of the translation machinery (Suarez et al., 2006). In *B. bovis* and *B. bigemina* the *ef-1α* locus consists in two identical *ef-1α* genes arranged in a head to head orientation separated by a 1.4 kb IG. Apparently, the IG contains two independent promoters (*ef-1α* IG-A and *ef-1α* IG-B) regulating bidirectional transcription of the two *ef-1α* open reading frames (ORFs) (De Koning-Ward et al., 1999; Silva et al., 2016; Suarez et al., 2006; Vinkenoog et al., 1998). Recently, it was found that promoters previously identified in *B. bovis ef-1α* IG region are also functional in *B. bigemina* (Silva et al., 2016). Likewise, interchangeable cross-species functional promoters, such as the *ef-1α* IG-B region have been identified between *B. gibsoni* and *B. bovis* (Liu et al., 2017a).

Currently, there are no identified promoter regions in the *B. ovis* genome sequence, thus in order to advance in the development of a stable transfection system for this parasite, the present study identified the functionality of two heterologous promoters to drive expression of a reporter gene, the *ef-1α* IG-B region from *B. bovis* and *B. ovata*.

2. Material and methods

2.1. *In vitro culture of Babesia ovis*

B. ovis in vitro culture was maintained in biosafety level 2 facilities at Instituto de Higiene e Medicina Tropical (IHMT), following an adapted protocol from Vega et al (1985). Briefly, cryopreserved *B. ovis* (Israeli strain) infected red blood cells (iRBC) at 8% parasitemia were used to initiate the culture. Parasites were cultured in 10% (vol/vol) defibrinated lamb erythrocytes (Biorabbit, Lisbon, Portugal) maintained in a HEPES-buffered Medium 199 (1x) containing 20% lamb serum (Gibco, Thermo Fischer Scientific), using a modular incubation chamber with a microaerophilic atmosphere (5% CO_2, 2% O_2 and 93% N_2) at 37 °C, as described (Horta et al., 2014). On a daily basis, 70% of the medium was replaced and culture parasitemia was monitored by preparation of thin blood smears stained with Hemacolor® Rapid staining of blood smear (EMD Millipore, Darmstadt, Germany). Intraerythrocytic parasites were observed under a 400× original magnification of a Motic BA210 LED trinocular compound microscope.

2.2. *Babesia ovis DNA extraction*

Parasite culture was centrifuged for 5 min at 200 × *g*, iRBCs pellet was resuspended in 500 μg mL^{-1} trypsin in phosphate buffered saline buffer (PBS) and incubated at 37 °C for 20 min. Complete medium was added in a 1:1 proportion to the pellet and mechanical lysis of erythrocytes was performed by ten passages through a 26-gauge needle. The suspension was centrifuged for 5 min/1500 × *g*, to retrieve the released *B. ovis* in the supernatant. Genomic DNA extraction was proceeded using NZY Blood gDNA Isolation Kit (NZYTech, Lisboa, Portugal), following manufacturers' instructions.

2.3. Plasmid constructs for transfection

Transient transfection constructs with *ef-1α-B* promoter region from *B. bovis* (pBbovis-luc) and *B. ovata* (pBovata-luc) (Fig. 1a) were developed based on transient transfection constructs previously used to transfect *B. bovis* (pBrfp-bsd) and *B. ovata* (*ef1-α* IG2), respectively (Asada et al., 2015; Hakimi et al., 2016)

To obtain plasmid pBbovis-luc, a luc-3'rap-1 cassette containing the *firefly luciferase* (*luc*) gene and the 3' *rhoptry associated protein-1* (*rap-1)* terminator sequence from *B. bovis* was constructed. The *luc* gene and the 3' *rap-1* terminator sequence were PCR amplified with primers PF-luc-EcoRV/ PR-luc-BglII and PF-bovis-rap-1-BglII/ PR-bovis-rap-1-BamHI from pLVX-TetOne™-Puro-Luc (Clontech, Mountain View, CA, USA) and from pBrfp-bsd, respectively. The two PCR products were digested with *Bgl*II restriction enzyme and ligated to generate the luc-3'rap-1 cassette. In a second step, plasmid pBrfp-bsd, containing the *ef-1α-B* promoter region from *B. bovis*, was restricted with *Eco*RV and *Bam*HI, which removes the reporter-selection cassette and the 3' *rap-1* terminator region from *B. bovis*. The linearized vector was then extracted and purified from an agarose gel and ligated to the luc-3'rap-1 cassette previously amplified by PCR with primers PF-luc-EcoRV/ PR-bovis-rap-1-BamHI and restricted with *Eco*RV and *Bam*HI. For the development of pBovata-luc, a plasmid carrying the *ef-1α* IG-B region from *B. ovata* (pBef1αIG2), was used as backbone. First, it was digested with *Eco*RV and *Eco*RI restriction enzymes which remove the reporter cassette, and the resulting linearized vector was ligated to the digested *luc* gene PCR amplified from pLVX-TetOne™-Puro-Luc (Clontech) with primers PF-luc-EcoRV/ PR-luc-EcoRI.

A promoterless plasmid was also constructed to use as negative control. Briefly, pBovata-luc was digested with *Hind*III restriction enzyme to excise the promoter sequence and was then re-circularized with T4 DNA ligase.

Plasmid constructs were transformed into *E. coli* JM109 competent cells (Stratagene, La Jolla, CA) as described previously (Sambrook and Russell, 2001) and the recombinant cells were selected from Luria–Bertani (LB) agar supplemented with ampicillin (100 μg mL^{-1}). Plasmids were extracted from the *E. coli* cells grown in LB broth supplemented with ampicillin at 37 °C, and purified using Qiagen Plasmid Midi Kit (Qiagen, Hilden, Germany) following manufacturers' instructions. All constructs were verified by Sanger sequencing at StabVida (Lisbon, Portugal) using appropriate primers (Table1)

Table 1
List of primers used for development of transfection constructs, for sequencing and to assess transfection efficiency.

Primer Name	Sequence (5′→3′)	Reference
Primers for transient transfection constructs		
PR-luc-***Bgl*II**	GCG**AGATCT**TTACAATTTGGACTTTCCG	This study
PF-luc-***Eco*RV**	GCC**GATATC**ATGGAAGACGCCAAAAAC	
PR-luc-***Eco*RI**	CGC**GAATTC**TTACAATTTGGACTTTCCGC	
PF-bovis-rap-1-***Bgl*II**	GCC**AGATCT**GATGAGATGCGTTTATAATGG	
PR-bovis-rap-1-***Bam*HI**	CGC**GGATCC**CCTACGAACGATATGTCAAAG	
Primers for sequencing		
PR-luc-seq.	CGCCGCCGTTGTTGTTTTGG	This study
PF-luc-seq.	CAGCCCATATCGTTTCATAGC	
M13F-pUC (-40)	GTTTTCCCAGTCACGAC	
Primers for qPCR to evaluate transfection efficiency		
PF-BoSPD	TAATGACGCAGACCTGATGG	Erster et al. (2016b)
PR-BoSPD	GTTTGATCACCCTCGGAAAC	
PF-qPCR-luc	GCTGGGCGTTAATCAGAGAG	This study
PR-qPCR-luc	GTGTTCGTCTTCGTCCCAGT	

Restriction enzyme sites are shown in **bold.**

2.4. *Babesia ovis* transfection

B. ovis iRBCs with PPE ~6% were centrifuged at $185 \times g$ for 10 min. The supernatant was discarded, and cell pellets were washed twice, first with PBS and then with Cytomix buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4, 10 mM KH_2PO_4, 25 mM HEPES, pH 7.6, 2 mM EGTA, 5 mM $MgCl_2$, 100 μg/ml bovine serum albumin, and 1 mM hypoxanthine), a protocol adapted from Asada et al. (2012). For transfection, two different protocols of iRBC preparation were conducted accordingly to the transfection kit in use. For the Basic Parasite Nucleofector™ Kit 2 (Lonza, Basel, Switzerland), 20 μg of circular plasmid in elution buffer were mixed with Nucleofector™ Basic Solution for Parasites to a final volume of 100 μL, which was used to resuspend 100 μL of washed parasite-iRBCs. For the Human T-cell Nucleofector™ Kit (Lonza), 20 μg or 50 μg of circular plasmid in elution buffer were mixed with Cytomix buffer to make up a volume of 50 μL and added to 50 μL of human T-cell buffer. Similarly, this mixture was used to resuspend 100 μL of washed parasite-iRBCs and nucleofection was carried out in a Nucleofector 2b Device with a v-024 program (Amaxa Biosystems, Cologne, Germany). Afterwards, transfected parasite-iRBCs were immediately transferred to 1 mL culture containing 10% ovine RBCs and cultured as described in Section 2.1.

2.5. Chemiluminescence quantification

Promoter activity was evaluated by luciferase assays at 24 h, 48 h and 72 h after transfection, using ONE-Glo™ Luciferase Assay System (Promega, Madison, Wisconsin, USA) following manufacturer´s instructions. Briefly, 100 μL of ONE-Glo™ Luciferase Assay Reagent was added to 100 μL of transfected *B. ovis* culture and this mixture was incubated at room temperature for 5 min before reading chemiluminescence, to ensure complete cell lysis. Luciferase assays were performed in triplicates in a Corning® Costar 96-well white plate (Corning Costar) and chemiluminescence was measured for a 2 s integration interval in a GloMax-Multi + Detection System (Promega). The results are from three independent transfections of each plasmid construct.

2.6. Quantitative real-time PCR

A quantitative real-time PCR (qPCR) was developed to assess the copy numbers of *luc* and *B. ovis surface protein D* (*BoSPD*) genes in the transfected *B. ovis* parasites using the sets of primers presented in Table 1, following the minimum information for publication of qPCR experiments (MIQE) guidelines (Bustin et al., 2009). Transfection efficiency is presented as the ratio of plasmid/genome copies (*luc/ BoSPD* copies), as previously published (Silva et al., 2016; Suarez et al., 2004).

The qPCRs were carried out in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, CA, USA) and the cycling conditions comprised an enzyme activation step at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 10 s and annealing/extension at 51 °C for 15 s and 60 °C for 15 s for *luc* and 54.5 °C for 30 s for *BoSPD.* To confirm amplification specificity for *luc* and *BoSPD*, a melting curve was run (55 °C – 95 °C; 0.5 °C/s melt rates). Reactions were performed in triplicate to a final volume of 10 μL, with 5 μL iTaq™ Universal SYBR® Green Supermix (Bio-Rad, CA, USA), with each pair of primers at 500 nM and 800 nM for *luc* and *BoSPD* amplification, respectively, 1 μL of DNA and nuclease-free water to achieve the final volume. Negative controls were prepared with no template. Standard curves were prepared for each gene with 10-fold serial dilutions of a synthetized gBlocks® Gene Fragment (Integrated DNA Technologies, Leuven, Belgium) containing their target sequences. These curves were obtained by plotting the threshold cycle (Cq) *versus* the natural log of concentration (ng μL^{-1}) and used to determine qPCR reactions efficiency (analytical sensitivity) and for quantification of *luc* and *BoSPD*. Copy numbers of *luc* and *BoSPD* genes were calculated based on the following formula, assuming that *BoSPD* gene only has one copy *per* genome, where X represents the amount of amplicon (ng):

$$\text{Copy number} = \frac{\text{X ng} \times 6.0221 \times 10^{23}\,\text{molecules/mols}}{\text{Amplicon molecular weight} \times 1 \times 10^{9}\,\text{ng/g}}$$

2.7. Statistical analysis

All the data were plotted as the mean ± standard deviation, calculated from the triplicate samples. Statistical significance was determined using GraphPad Prism6 (GraphPad Software Inc., San Diego, CA, USA). An unpaired two sample *t* test was performed to compare data concerning transfection parameters optimization. The luminescence and ratio of plasmid/ "genome copies" values were evaluated using a one-way ANOVA analysis followed by Dunnett's or Tukey's multiple comparison test to determine statistical significance. For statistical analysis of all data $P < 0.05$ was the lowest acceptable threshold for significance.

3. Results and discussion

3.1. Optimization of Babesia ovis transfection parameters: nucleofection solution

Until now, experimental conditions had not been determined for short term transfection of *B. ovis,* with no pulse condition or nucleofection solution recommended by Amaxa for nucleofection of *Babesia* spp. The present work initiated with the selection of Amaxa nucleofector program V-024 with transfection of 20 μg of plasmid, as used previously for *B. ovata* transfection (Hakimi et al., 2016), and the results of nucleofection solution optimization are shown in Fig. 1b and c.

Transfection was performed with both plasmids (pBbovis-luc or pBovata-luc) since no information regarding the ability of heterologous promoters to promote exogenous gene expression in *B. ovis* was available. The effect of transfecting *B. ovis* with either "Human T-cell" or "Parasite" solution was observed in the luciferase activity and on parasite viability 24, 48 and 72 h post-transfection.

The observed luciferase activity indicated that transfection of 20 μg of plasmid using the program V-024 was suitable and that both "Human T-Cell" and "Parasite" solutions were candidates to proceed with the transfection experiments (Fig. 1b). In accordance to a previous study in which *B. bovis* transient transfection using two different buffers, the "Plasmodium 88A6″, herein named "Parasite", and the "Human T-cell", led to no significant differences in luciferase expression (Suarez and McElwain, 2008).

Regarding the impact of each transfection buffer in the observable PPE, no statistically significant differences were found 24, 48 and 72 h after transfection with different buffers. Nonetheless, whereas for the parasites transfected in the "Parasite" buffer there is no visible change in PPE along the 72 h, those transfected in "T-cell" solution do increase (not to a statistically significant degree) (Fig. 1c). Contrastingly, in a previous study, transfection of *B. bovis* free merozoites with either of the solution buffers led to a noticeable increase in PPE between 24 h and 48 h post-transfection (Suarez and McElwain, 2008). Thus, one possible reason for the differences observed in the present study might be a higher protective effect of the "Human T-cell" solution in the erythrocyte membrane. This assumption is further supported by the increased presence of erythrocyte lysis in stained blood smears after transfection with the "Parasite" solution (data not shown). One might also suggest that depletion of the intraerythrocytic ATP levels, essential for *Babesia* spp. growth, was higher when using a transfection buffer that failed to exert a more protective effect during the process of erythrocyte membrane permeabilization (Caro et al., 2012; Kim et al., 2015). It is also possible to observe that the nucleofection process has a clear detrimental effect on parasite survival (initial PPE was of ∼ 6%) (Fig. 1c), an impact described previously as being significant but inferior to the conventional electroporation method (Burkard et al., 2007; Jongco et al., 2006; Sauerwein et al., 2005; Suarez and McElwain, 2008).

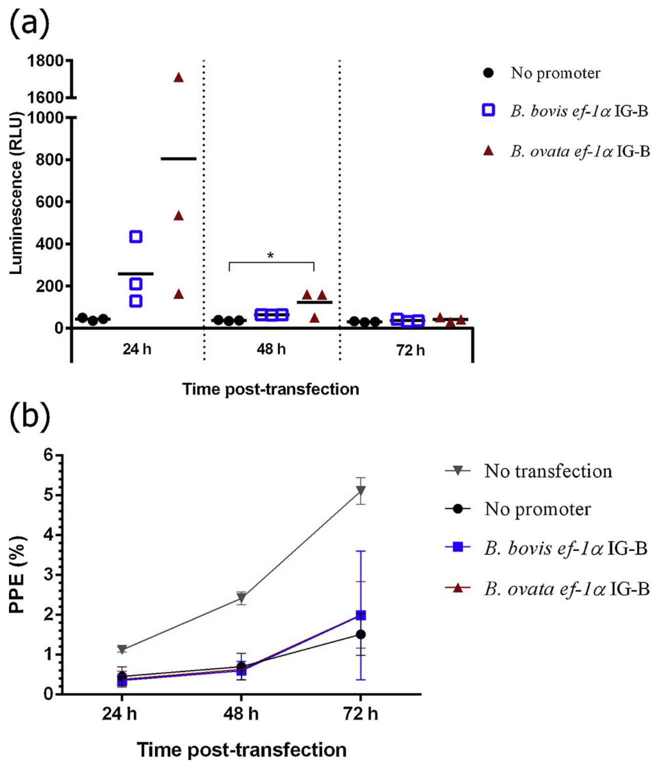

Fig. 2. Evaluation of *Babesia bovis and Babesia ovata*'s *elongation factor-1alpha* intergenic region-B heterologous promoter activity in *Babesia ovis.* (A) Evaluation of the promoter activity of *ef-1α* IG-B from *B. bovis* and *B.ovata* driving expression of a reporter luciferase 24, 48 and 72 h post *B. ovis* transfection. A negative control was performed through transfection of a promoterless plasmid (No promoter). A negative control to the transfection procedure was performed through maintaining a culture of non-transfected parasites (No transfection). Data is expressed as the mean of the obtained RLUs of three independent transfections with each plasmid (*B. bovis ef-1α* IG-B, *B. ovata ef-1α* IG-B and No promoter) + SD. RLU: Relative luciferase units. Data was compared using One-way ANOVA analysis, with no significant differences detected ($F_{(2,6)} = 1.989$, $P = 0.2174$ at 24 h, $F_{(2,6)} = 1.306$, $P = 0.3383$ at 48 h and $F_{(2,6)} = 1.73$, $P = 0.2552$ at 72 h), followed by Dunnett's multiple comparison test with significant differences detected (*$P < 0.05$) (B) Percentage of parasitized erythrocytes (PPE) 24, 48 and 72 h post *B. ovis* transfection. Data is expressed as the mean of three independent transfections ± SD. Data was compared using One-way ANOVA analysis, with no significant differences detected in PPE after transfection with different plasmid constructs ($F_{(2,6)} = 0.2083$, $P = 0.8176$ at 24 h, $F_{(2,6)} = 0.1434$, $P = 0.8693$ at 48 h and $F_{(2,6)} = 0.1942$, $P = 0.8284$ at 72 h), followed by a Tukey's multiple comparison test with no significant differences detected ($P < 0.05$).

Considering the overall results and previous nucleofection studies of *B. bovis* and *B. ovata* iRBCs, the Human T-cell solution was selected for further experiments (Asada et al., 2015, 2012; Hakimi et al., 2016).

3.2. Kinetics of luciferase expression after transfection of B. ovis iRBCs

Luciferase expression was evaluated at 24, 48 and 72 h post *B. ovis* transfection with the transient plasmid constructs (Fig. 1a). For the conditions undertaken in the present study, the maximum *luc* expression was observed at 24 h post-transfection with either pBbovis-luc or pBovata-luc and, after 48 h, it decreased to 24.5% and 15.3% of the peak value obtained for each plasmid, respectively. The obtained luminescence at 72 h post-transfection was negligible and the negative control (No promoter) presented a background luminescence identical to the observed in the empty wells at all time points analyzed (Fig. 2a). Recent studies with *Babesia* spp. using either *ef-1α* IG heterologous or homologous promoters to drive *luc* expression have also reported the peak at 24 h post-transfection and the decrease of luminescence along the 72 h period (Liu et al., 2017b; Silva et al., 2016). In contrast, a study in *B. ovata* with homologous *actin* promoter driving expression of a reporter luciferase shows a peak of luciferase activity 48 h post-transfection, an expression that is maintained until 96 h (Hakimi et al., 2016). A similar disparity had been observed previously for *B. bovis* between *ef-1α* IG and *rap-1* IG promoters driving *luc* expression and it was attributed to differences in the regulation of those genes' expression (Suarez et al., 2004; Suarez and McElwain, 2008).

A common feature between transient transfection experiments is that the expression kinetics of the *luc* gene is a reflect of the transient nature of the genetic material. After transfection, plasmid maintenance depends on its ability to replicate along with the host cell and of a selective pressure. In *Babesia* spp. a transgene can be maintained through genome integration, which occurs exclusively by mechanisms of homologous recombination, or as extra-chromosomal replicating episomes (Asada et al., 2015, 2012; Suarez et al., 2015). Moreover, the inexistence of a selective pressure to maintain the plasmid will cause uneven segregation between daughter merozoites and consequent loss of the plasmid (Van Dijk et al., 1997). This phenomenon has been observed through the inversely proportional relationship between PPE post-transfection and luminescence values. While this has been reported in previous *Babesia* spp. transfection studies (Liu et al., 2017b; Suarez and McElwain, 2008), herein the dramatic decay of luciferase activity levels from 24 to 48 h is coupled to a PPE that does not change significantly ($P > 0.05$) (Fig. 2b). The half-life of luciferase enzyme in mammalian living cells is quite short (≈ 2 h) (Ignowski and Schaffer, 2004) thus, the luminescence values obtained at 48 h are conceivably resultant of *luc* gene transcription until that time point. The differences observed in the kinetics of luciferase in *B. ovis* are probably related with the different growth rate of these different species or the difference in the maintenance period of plasmid constructs in various *Babesia* species (Kim and Eberwine, 2010; Liu et al., 2017b; Suarez and McElwain, 2008).

3.3. *The elongation factor-1alpha intergenic region-B from Babesia ovata has heterologous promoter activity in Babesia ovis*

The promoter activity of *ef-1α* IG-B region from *B. bovis* and *B. ovata* was tested using luciferase assays. Both promoter regions used in the present study had been previously validated concerning their ability to drive expression of a reporter gene in their respective species (Asada et al., 2015; Hakimi et al., 2016). Moreover, initial experiments to optimize the transfection parameters already indicated that the *B. ovis* transcriptional machinery was able to recognize both promoter regions and drive expression of the reporter luciferase (Fig. 1b).

Herein, we demonstrate that both *B. ovata* and *B. bovis's ef-1α* IG-B regions are recognized by the *B. ovis* transcriptional machinery, being able to drive expression of a reporter *luc*. Though, only the *ef-1α-B* promoter region from *B. ovata* resulted in statistically significantly higher luminescence values in comparison to the control (No promoter) at 48 h post transfection ($P < 0.05$) whereas the luciferase activity values from *B. bovis*' *ef-1α-B* promoter region were not significantly different in comparison to the control (Fig. 2a). Furthermore, it is noticeable that the *ef-1α*-B promoter region from *B. ovata* can induce expression of higher levels of luciferase indicating that *B. ovis* transcription factors have a higher binding affinity towards motifs existent in *B. ovata ef-1α* IG-B region (Tretina et al., 2016). These transcription factors can be either sequence-specific DNA-binding proteins involved in specific regulation of a certain gene or more general, related with RNA polymerase II components required for transcription initiation. However, absence of knowledge regarding *B. ovis cis*-regulatory motifs does not allow to infer which binding factors might be weighing in the presently obtained results (Mullapudi et al., 2009; Yamagishi et al., 2014). Recently, it was attempted to transfect *B. ovata* with a plasmid having *B. bovis ef-1α* IG-B region, but this region was not able to induce expression of the reporter luciferase, a fact attributed to a lack of recognition by *B. ovata* transcriptional or translational machinery (Hakimi et al., 2016). Therefore, one can suggest that the sequence of *B. bovis ef-1α* IG-B does not have certain motifs necessary for transcription factor recognition by *B. ovata,* being probably quite different from the *B. ovata* homologous *ef-1α* IG-B sequence. Thus, this supports the differences observed in the *luc* expression of these promoter regions in *B. ovis.*

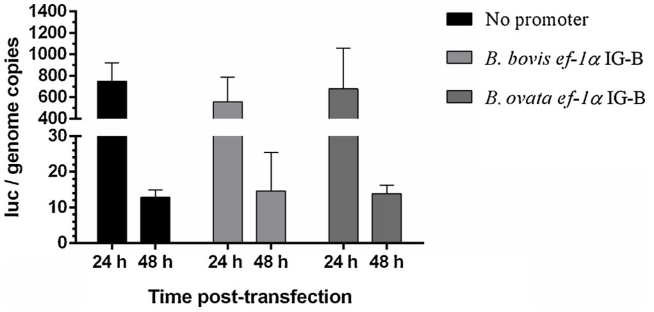

Fig. 3. Efficiency of *Babesia ovis* transient transfection. Evaluation of transfection efficiency of the plasmid constructs by the *luc*/ "genome copies" ratio. A qPCR was carried out to assess the number of plasmid DNA copies *per* "genome" 24 h and 48 h post-transfection targeting *luc* and *BoSPD* genes. The ratio of plasmid /"genome copies" was calculated assuming that *BoSPD* has one copy per genome. Data was compared using One-way ANOVA analysis, with no significant differences detected in transfection efficiency of the plasmid constructs at either 24 h ($F_{(2,6)} = 0.3695$, $P = 0.7058$) or 48 h ($F_{(2,5)} = 0.04742$, $P = 0.9541$), followed by a Tukey's multiple comparison test with no significant differences detected ($P < 0.05$).

As previously mentioned, different growth rates can result in distinct *luc* expression kinetics. Analysis of PPE was performed at 24, 48 and 72 h post-nucleofection and no statistically significant differences were found in *B. ovis* transfected parasites ($F_{(2,6)} = 0.2083$, $P = 0.8176$ at 24 h, $F_{(2,6)} = 0.1434$, $P = 0.8693$ at 48 h and $F_{(2,6)} = 0.1942$, $P = 0.8284$ at 72 h), indicating that parasites are similarly affected by the nucleofection process regardless of the plasmid construct transfected (Fig. 2b). Distinct transfection efficiencies between the transfected plasmids may also explain the differences observed in *luc* expression (Fig. 3). Transfection efficiency was calculated as the ratio between *luc* and *BoSPD* genes after extracting gDNA from the parasite culture at 24 and 48 h post-transfection. The *luc* gene sequence is unique to the transfection plasmid while the *BoSPD* gene sequence is unique to the parasite and, for the current study, we assumed the presence of one *BoSPD* copy *per* genome. This evaluation has been used in previous transfection studies and is accurate in quantifying the plasmid DNA that entered the parasite (Silva et al., 2016; Suarez et al., 2004). The qPCR demonstrated similar efficiencies between transfection with the different plasmid constructs both at 24 h ($F_{(2,6)} = 0.3695$, $P = 0.7058$) and 48 h ($F_{(2,5)} = 0.04742$, $P = 0.9541$) (Fig. 3). Strikingly, the *luc* / "genome" ratio values detected at 24 h post transfection are in the range of those observed for high copy number plasmids after *E. coli* transformation (Anindyajati et al., 2016).

In summary, it is possible to preclude the hypothesis that differences in the obtained luminescence values are related with differences in PPE after transfection or transfection efficiency itself. Our data suggest that *B. ovis* transcriptional factors have a stronger affinity for *B. ovata* than for the *B. bovis ef-1α-B* promoter sequence. Curiously, phylogenetic analysis based on the *18S ribosomal RNA* (*rRNA*) gene have placed *B. ovis* assembling with *B. bovis* in a group of moderate support, quite separate from the group containing *B. ovata* (Schnittger et al., 2012).

The present work sustains previous reports of interspecies promoter activity between *Babesia* spp. Interestingly, in a previous study, the *ef-*

1α IG heterologous promoter sequence showed to be more efficient driving expression of the reporter *luc* than the homologous promoter in *B. bigemina*, and similar results were found in the latter study with *B. gibsoni* 5' *act* being more active than the homologous promoter in *B. bovis* (Liu et al., 2017a; Silva et al., 2016). Given the absence of *B. ovis ef-1α* IG-B region nucleotide sequence, the interchangeable cross-species activity of this promoter region could not be explored. Nevertheless, the *ef-1α-B* promoter from *B. ovata* was found suitable for driving exogenous gene expression in the context of a stable transfection system, and a study in *B. bovis* suggests that the use of heterologous regulatory regions such as cross-species functional promoters increases the specificity of the genome integration event (Suarez et al., 2015).

The present results also show that both *B. bovis* and *B. ovata* 3' *rap-1* terminator regions have a cross-species function in *B. ovis.* The cross-species function of this region had only been described once before with *B. bovis* 3' *rap-1* terminator region being functional in *B. gibsoni* (Liu et al., 2017a). A study that analysed *rap-1* IG regions from *B. bovis*, *B. bigemina*, *B. canis* and *B. ovis* concluded that the sequence conservation between species was relatively low with the highest sequence similarity (42% identity) being found between *B. ovis* and *B. bovis* sequences (Suarez et al., 1998). It appears that both *B. bovis* and *B. ovata rap-1* IG regions have the necessary termination signals to be functional in *B. ovis.*

4. Conclusions

B. ovis was successfully transfected for the first time and, while only a preliminary study, here we present an initial demonstration of proof-of-principle for *B. ovis* genetic manipulation. The successful use of constructs bearing heterologous promoters will facilitate the development of transgenic and knockout parasites through integration-dependent transfection. At least for *B. ovis* and *B. ovata*, a common stable transfection platform appears to be feasible. Currently, efforts are being conducted to find suitable selectable markers for *B. ovis*, supporting the design of plasmids aiming either genome integration or episomal maintenance. These novel tools for genetic manipulation will certainly clarify functions of proteins implicated in virulence and accelerate identification of new candidates for vaccine development.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgements

Authors would like to acknowledge Fundação para a Ciência e Tecnologia (FCT) for funds to GHTM - UID/Multi/04413/2013. We are thankful to Francisco Olivença from iMed.ULisboa for outstanding technical assistance in the development of the transient transfection constructs. A special thanks to Abel Oliva from Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa and Varda Shkap, from Kimron Veterinary Institute, Israel, for providing the Israeli strain of *B. ovis.*

References

Anindyajati, Anita Artarini, A., Riani, C., Retnoningrum, D.S., 2016. Plasmid copy number determination by quantitative polymerase chain reaction. Sci. Pharm. 84, 89–101. https://doi.org/10.3797/scipharm.ISP.2015.02.

Antunes, S., Rosa, C., Couto, J., Ferrolho, J., Domingos, A., 2017. Deciphering *Babesia*-vector interactions. Front. Cell. Infect. Microbiol. 7, 1–8. https://doi.org/10.3389/fcimb.2017.00429.

Aouadi, A., Leulmi, H., Boucheikhchoukh, M., Benakhla, A., Raoult, D., Parola, P., 2017. Molecular evidence of tick-borne hemoprotozoan-parasites (*Theileria ovis* and *Babesia ovis*) and bacteria in ticks and blood from small ruminants in Northern Algeria. Comp. Immunol. Microbiol. Infect. Dis. 50, 34–39. https://doi.org/10.1016/j.cimid.2016.11.008.

Asada, M., Tanaka, M., Goto, Y., Yokoyama, N., Inoue, N., Kawazu, S.I., 2012. Stable expression of green fluorescent protein and targeted disruption of *thioredoxin peroxidase-1* gene in *Babesia bovis* with the WR99210/*dhfr* selection system. Mol. Biochem. Parasitol. 181, 162–170. https://doi.org/10.1016/j.molbiopara.2011.11.001.

Asada, M., Yahata, K., Hakimi, H., Yokoyama, N., Igarashi, I., Kaneko, O., Suarez, C.E., Kawazu, S.I., 2015. Transfection of *Babesia bovis* by double selection with WR99210 and blasticidin-S and its application for functional analysis of *thioredoxin peroxidase-1*. PLoS One 10, 1–13. https://doi.org/10.1371/journal.pone.0125993.

Belloli, C., Lai, O.R., Ormas, P., Zizzadoro, C., Sasso, G., Crescenzo, G., 2006. Pharmacokinetics and mammary elimination of imidocarb in sheep and goats. J. Dairy Sci. 89, 2465–2472. https://doi.org/10.3168/jds.S0022-0302(06)72320-7.

Burkard, G., Fragoso, C.M., Roditi, I., 2007. Highly efficient stable transformation of bloodstream forms of *Trypanosoma brucei*. Mol. Biochem. Parasitol. 153, 220–223. https://doi.org/10.1016/j.molbiopara.2007.02.008.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622. https://doi.org/10.1373/clinchem.2008.112797.

Caro, F., Miller, M.G., DeRisi, J.L., 2012. Plate-based transfection and culturing technique for genetic manipulation of *Plasmodium falciparum*. Malar. J. 11, 1–10. https://doi.org/10.1186/1475-2875-11-22.

De Koning-Ward, T.F., Sperança, M.A., Waters, A.P., Janse, C.J., 1999. Analysis of stage specificity of promoters in *Plasmodium berghei* using luciferase as a reporter. Mol. Biochem. Parasitol. 100, 141–146. https://doi.org/10.1016/S0166-6851(99)00042-0.

Ekici, O.D., Sevinc, F., Isik, N., 2012. Instability of ovine babesiosis in an endemic area in Turkey. Vet. Parasitol. 188, 372–375. https://doi.org/10.1016/j.vetpar.2012.04.001.

Enayati, A.A., Asgarian, F., Amouei, A., Sharif, M., Mortazavi, H., Boujhmehrani, H., Hemingway, J., 2010. Pyrethroid insecticide resistance in *Rhipicephalus bursa* (Acari, Ixodidae). Pest. Biochem. Physiol. 97, 243–248. https://doi.org/10.1016/j.pestbp.2010.03.003.

Erster, O., Roth, A., Wolkomirsky, R., Leibovich, B., Savitzky, I., Shkap, V., 2016a. Transmission of *Babesia ovis* by different *Rhipicephalus* bursa developmental stages and infected blood injection. Ticks Tick. Borne. Dis. 7, 13–19. https://doi.org/10.1016/j.ttbdis.2015.07.017.

Erster, O., Roth, A., Wollkomirsky, R., Leibovich, B., Savitzky, I., Zamir, S., Molad, T., Shkap, V., 2016b. Quantitative analysis of *Babesia ovis* infection in sheep and ticks. Vet. Parasitol. 221, 39–45. https://doi.org/10.1016/j.vetpar.2016.03.004.

Hakimi, H., Yamagishi, J., Kegawa, Y., Kaneko, O., Kawazu, S., Asada, M., 2016. Establishment of transient and stable transfection systems for *Babesia ovata*. Parasit. Vectors 9, 171. https://doi.org/10.1186/s13071-016-1439-z.

Horta, S., Barreto, M.C., Pepe, A., Campos, J., Oliva, A., 2014. Highly sensitive method for diagnosis of subclinical *B. ovis* infection. Ticks Tick. Borne Dis. 5, 902–906. https://doi.org/10.1016/j.ttbdis.2014.07.005.

Ignowski, J.M., Schaffer, D.V., 2004. Kinetic analysis and modeling of firefly luciferase as a quantitative reporter gene in live mammalian cells. Biotechnol. Bioeng. 86, 827–834. https://doi.org/10.1002/bit.20059.

Jongco, A.M., Ting, L.M., Thathy, V., Mota, M.M., Kim, K., 2006. Improved transfection and new selectable markers for the rodent malaria parasite *Plasmodium yoelii*. Mol. Biochem. Parasitol. 146, 242–250. https://doi.org/10.1016/j.molbiopara.2006.01.001.

Kim, K., Park, Y., Kim, Y., Park, H., Cho, S.-H., Lee, S.-E., Lee, W.-J., Hong, S.-H., 2015. Characterizations of individual mouse red blood cells parasitized by *Babesia microti* using 3-D holographic microscopy. Sci. Rep. 5, 1–11. https://doi.org/10.1038/srep10827.

Kim, T.K., Eberwine, J.H., 2010. Mammalian cell transfection: the present and the future. Anal. Bioanal. Chem. 397, 3173–3178. https://doi.org/10.1007/s00216-010-3821-6.

Liu, M., Adjou Moumouni, P.F., Asada, M., Hakimi, H., Masatani, T., Vudriko, P., Lee, S.H., Kawazu, S.I., Yamagishi, J., Xuan, X., 2018. Establishment of a stable transfection system for genetic manipulation of *Babesia gibsoni*. Parasit. Vectors 11, 1–6. https://doi.org/10.1186/s13071-018-2853-1.

Liu, M., Adjou Moumouni, P.F., Cao, S., Asada, M., Wang, G., Gao, Y., Guo, H., Li, J., Vudriko, P., Efstratiou, A., Ringo, A.E., Lee, S.H., Hakimi, H., Masatani, T., Sunaga, F., Kawazu, Sichiro, Yamagishi, J., Jia, L., Inoue, N., Xuan, X., 2017a. Identification and characterization of interchangeable cross-species functional promoters between *Babesia gibsoni* and *Babesia bovis*. Ticks Tick. Borne. Dis. 9, 330–333. https://doi.org/10.1016/j.ttbdis.2017.11.008.

Liu, M., Asada, M., Cao, S., Franck, P., Moumouni, A., Vudriko, P., Efstratiou, A., Hakimi, H., Masatani, T., Sunaga, F., Kawazu, S., 2017b. Transient transfection of intraerythrocytic *Babesia gibsoni* using *elongation factor-1 alpha* promoter. Mol. Biochem. Parasitol. 216, 56–59. https://doi.org/10.1016/j.molbiopara.2017.07.003.

McHardy, N., Woollon, R.M., Clampitt, R.B., James, J.A., Crawley, R.J., 1986. Efficacy, toxicity and metabolism of imidocarb dipropionate in the treatment of *Babesia ovis* infection in sheep. Res. Vet. Sci. 41, 14–20.

Mullapudi, N., Joseph, S.J., Kissinger, J.C., 2009. Identification and functional characterization of *cis*-regulatory elements in the apicomplexan parasite *Toxoplasma gondii*. Genome Biol. 10. https://doi.org/10.1186/gb-2009-10-4-r34.

Oldiges, D.P., Laughery, J.M., Tagliari, N.J., Leite Filho, R.V., Davis, W.C., da Silva Vaz, I., Termignoni, C., Knowles, D.P., Suarez, C.E., 2016. Transfected *Babesia bovis* expressing a tick GST as a live vector vaccine. PLoS Negl. Trop. Dis. 10, e0005152. https://doi.org/10.1371/journal.pntd.0005152.

Ranjbar-Bahadori, S., Eckert, B., Omidian, Z., Shirazi, N.S., Shayan, P., 2012. *Babesia ovis* as the main causative agent of sheep babesiosis in Iran. Parasitol. Res. 110, 1531–1536. https://doi.org/10.1007/s00436-011-2658-z.

Sambrook, J., Russell, D., 2001. Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory Press, NY.

Santos, Pa, Sant'Anna, J.R., Franco, C.C., Rosada, L.J., Esquissato, G.N., Castro-Prado, Ma, 2012. Induced mitotic homologous recombination by the babesicide imidocarb dipropionate in *Aspergillus nidulans* diploid cells. Genet. Mol. Res. 11, 1810–1818. https://doi.org/10.4238/2012.July.6.1.

Sauerwein, R.W., van Gemert, G.J., Thiel, C., Waters, A.P., Ramesar, J., Engelmann, S., Matuschewski, K., Janse, C.J., Mair, G.R., Franke-Fayard, B., 2005. High efficiency transfection of *Plasmodium berghei* facilitates novel selection procedures. Mol. Biochem. Parasitol. 145, 60–70. https://doi.org/10.1016/j.molbiopara.2005.09.007.

Schnittger, L., Rodriguez, A.E., Florin-Christensen, M., Morrison, Da., 2012. *Babesia:* a world emerging. Infect. Genet. Evol. 12, 1788–1809. https://doi.org/10.1016/j.meegid.2012.07.004.

Shayan, P., Hooshmand, E., Rahbari, S., Nabian, S., 2007. Determination of *Rhipicephalus* spp. as vectors for *Babesia ovis* in Iran. Parasitol. Res. 101, 1029–1033. https://doi.org/10.1007/s00436-007-0581-0.

Silva, M.G., Knowles, D.P., Mazuz, M.L., Cooke, B.M., Suarez, C.E., 2018. Stable transformation of *Babesia bigemina* and *Babesia bovis* using a single transfection plasmid. Sci. Rep. 8, 1–9. https://doi.org/10.1038/s41598-018-23010-4.

Silva, M.G., Knowles, D.P., Suarez, C.E., 2016. Identification of interchangeable cross-species function of *elongation factor-1 alpha* promoters in *Babesia bigemina* and *Babesia bovis*. Parasit. Vectors 9, 576. https://doi.org/10.1186/s13071-016-1859-9.

Suarez, C.E., Bishop, R.P., Alzan, H.F., Poole, W.a., Cooke, B.M., 2017. Advances in the application of genetic manipulation methods to apicomplexan parasites. Int. J. Parasitol. 47, 701–710. https://doi.org/10.1016/j.ijpara.2017.08.002.

Suarez, C.E., Johnson, W.C., Herndon, D.R., Laughery, J.M., Davis, W.C., 2015. Integration of a transfected gene into the genome of *Babesia bovis* occurs by legitimate homologous recombination mechanisms. Mol. Biochem. Parasitol. 202, 23–28. https://doi.org/10.1016/j.molbiopara.2015.09.003.

Suarez, C.E., McElwain, T.F., 2010. Transfection systems for *Babesia bovis:* a review of methods for the transient and stable expression of exogenous genes. Vet. Parasitol. 167, 205–215. https://doi.org/10.1016/j.vetpar.2009.09.022.

Suarez, C.E., McElwain, T.F., 2008. Transient transfection of purified *Babesia bovis* merozoites. Exp. Parasitol. 118, 498–504. https://doi.org/10.1016/j.exppara.2007.10.013.

Suarez, C.E., Norimine, J., Lacy, P., McElwain, T.F., 2006. Characterization and gene expression of *Babesia bovis elongation factor-1alpha*. Int. J. Parasitol. 36, 965–973. https://doi.org/10.1016/j.ijpara.2006.02.022.

Suarez, C.E., Palmer, G.H., Hötzel, I., Hines, S.a., McElwain, T.F., 1998. Sequence and functional analysis of the intergenic regions separating babesial rhoptry-associated protein-1 (*rap-1*) genes. Exp. Parasitol. 90, 189–194. https://doi.org/10.1006/expr.1998.4321.

Suarez, C.E., Palmer, G.H., LeRoith, T., Florin-Christensen, M., Crabb, B., McElwain, T.F., 2004. Intergenic regions in the *rhoptry associated protein-1* (*rap-1*) locus promote exogenous gene expression in *Babesia bovis*. Int. J. Parasitol. 34, 1177–1184. https://doi.org/10.1016/j.ijpara.2004.07.001.

Tretina, K., Pelle, R., Silva, J.C., 2016. Cis regulatory motifs and antisense transcriptional control in the apicomplexan *Theileria parva*. BMC Genomics 17, 1–13. https://doi.org/10.1186/s12864-016-2444-5.

Van Dijk, M.R., Vinkenoog, R., Ramesar, J., Vervenne, R.A.W., Waters, A.P., Janse, C.J., 1997. Replication, expression and segregation of plasmid-borne DNA in genetically transformed malaria parasites. Mol. Biochem. Parasitol. 86, 155–162. https://doi.org/10.1016/S0166-6851(97)02843-0.

Vega, C.A., Buening, G.M., Green, T.J., Carson, C.A., 1985. *In vitro* cultivation of *Babesia bigemina*. Am. J. Vet. Res. 46, 416–420.

Vinkenoog, R., Speranca, M.A., van Breemen, O., Ramesar, J., Williamson, D.H., Ross-MacDonald, P.B., Thomas, A.W., Janse, C.J., del Portillo, H.A., Waters, A.P., 1998. Malaria parasites contain two identical copies of an *elongation factor 1 alpha* gene. Mol. Biochem. Parasitol. 94, 1–12.

Yamagishi, J., Wakaguri, H., Yokoyama, N., Yamashita, R., Suzuki, Y., Xuan, X., Igarashi, I., 2014. The *Babesia bovis* gene and promoter model: an update from full-length EST analysis. BMC Genomics 15, 678. https://doi.org/10.1186/1471-2164-15-678.