Establishment of transient and stable transfection systems for genetic manipulation of *Babesia gibsoni*

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バベシア･ギブソニの遺伝子操作のための一過性及び安定したトランスフェクションシステムの確立

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Contents

Abbreviations and unit abbreviations

Abbreviations

B	BSD	Blasticidin
C	CMV	Cytomegalovirus
D	DNA	Deoxyribonucleic acid
E	EDTA	Ethylenediaminetetraacetic acid
	ef-1α	Elongation factor-1 alpha
	eGFP	Enhanced green fluorescent
G	GFP	Green fluorescent protein
	GI	Genome integrated
H	hdhfr	Human dihydrofolate reductase
	hpt	Hours post transfection
	hsp70	Heat shock protein 70
I	IC_{50}	Half maximal inhibitory concentration
	IG	Intergenic region
	iRBCs	Infected red blood cell
O	ORF	Open reading frame
P	pBS	pBluescript SK (+)
	PCR	Polymerase chain reaction
	pH	Potential of hydrogen
	PI	Propidium Iodide
R	rap-1	Rhoptry associated protein-1
	RBCs	Red blood cell
	RNA	Ribonucleic acid
	RPMI	Roswell Park Memorial Institute
	RT	Room temperature
S	SD	Standard deviation

	SV40	Simian Virus 40
T	TE	Tris EDTA
	tpx-1	Thioredoxin peroxidase-1
U	UTR	Untranslated region
W	WT	Wild type

Unit abbreviations

bp	base pair
kb	kilo base
µl	microliter
ml	milliliter
µg	microgram
nM	nanomolar
s	second
min	minute
h	hour
U	unit
%	percentage
°C	degree Celsius

General introduction

1. Babesiosis

Babesiosis is a tick-transmitted, zoonotic disease caused by hematotropic parasites of the genus *Babesia* (Marathe et al., 2005). *Babesia* parasites are the second most common and widespread blood parasites in the world, after trypanosomes, Babesia parasites consequently have considerable worldwide medical, economic and veterinary impact (Singh et al., 2014).

So far, more than 100 *Babesia* species have been described which infect many types of mammalian hosts, even in several kinds of birds (Chauvin et al., 2009). The host range of *B. microti* and *B. divergens* are wide, from small mammals to humans. Clinical symptoms of human babesiosis usually include intense parasitemia with neurological complications and sometimes fatal outcome (Skotarczak, 2008). *B. equi* (Formerly *Theileria equi*) and *B. caballi*, naturally found in horses, lead to an acute tick-borne hemolytic anemia in susceptible horses (Baldani et al., 2007; Malekifard et al., 2014). *B. bovis* and *B. bigemina* are pathogens of cattle and other wild ruminants, there is an acute hemolytic phase which is often fatal (Yu et al., 2013; Abdela and Jilo, 2016). *B. canis* and *B. gibsoni* are worldwide distributed, which are the most widespread *Babesia* species in dogs and lead to fever, jaundice, hemoglobinuria and anemia (Yao et al., 2014; Bhaskaran Ravi et al., 2016).

Babesia parasites require both of vertebrate and non-vertebrate host to maintain transmission cycles (Duh et al., 2001; Rudolf et al., 2005; Kusakisako et al., 2015). After the first report, the increasing reported cases of babesiosis may be due to the rise

in actual incidence, as well as the enhanced awareness of this disease. Despite of advances in prevention and diagnosis which result in the extensive research and a greater understanding of this disease, it still have significant economic and medical impacts (Homer et al., 2000).

2. Life cycle of *Babesia* parasites

Babesia parasites are vector-borne protozoa transmitted by arthropod, the life cycle of *Babesia* parasites are including asexual multiplication in vertebrate blood cells, sexual reproduction in the vector and consists of production of sporozoites in the salivary glands of the vector (Boustani and Gelfand, 1996; Chauvin et al., 2009). Thus, *Babesia* are considered to be a hemoprotozoan genus that is specifically adapted to use ticks as vectors (Gondard et al., 2017).

Babesia parasites have two hosts, the vertebrate host and tick vector. The network among ticks, vertebrates and parasites constitute complex system (Chauvin et al., 2009). Transmission of *Babesia* parasites from one host to another takes place through direct tick-host contact, normally, the contact is accomplished by the bite of *Babesia*-infected ticks, the natural transmission between the tick and host needs 2 weeks, which is depending on the species and stage of ticks (larva, nymph or adult). After the parasites were introduced to tick salivary as a sporozoite form, it may persist asymptomatically in the host for several years (Vannier et al., 2015; Scott and Scott, 2018).

3. Canine babesiosis

Canine babesiosis is a significant tick-borne disease caused by various species of the protozoan genus *Babesia*. Both the large and small forms of *Babesia* species (*B. canis*, *B. vogeli*, *B. rossi*, *B. gibsoni*, *B. conradae and B. vulpes*) infect dogs, and their transmission, clinical signs, treatment, geographical distribution and prognosis vary widely for each species (Halos et al., 2014; Solano-Gallego et al., 2016).

Clinical symptoms of canine babesiosis vary depending on the strains and species involved, and also the host's response to infection such as individual immune status, age and the presence of concurrent infections or other diseases (Birkenheuer et al., 1999; Jacobson, 2006). Hemolytic anemia with systemic inflammatory response and erythrocyte destruction, which may lead to organ dysfunction, account for most of the clinical signs observed in canine babesiosis (Baneth, 2018). Canine babesiosis onset is often acute with affected dogs suffering from fever and lethargy, thereafter may display clinical symptoms, such as hepatic, pulmonary, kidney or cerebral dysfunction, anemia and hemostatic abnormalities including electolyte imbalances and coagulation (Leisewitz et al., 2001; Eichenberger et al., 2016).

4. *Babesia gibsoni*

B. gibsoni is the most common causative agent of canine babesiosis in Asian countries (Liu et al., 2016), including Japan, Korea, Malaysia and Taiwan (Inokuma et al., 2004; Miyama et al., 2006; Lee et al., 2009; Mokhtar et al., 2013). Recently, *B. gibsoni* has also been reported in China (Cao et al., 2015; He et al., 2017; Lin et al., 2017; Zheng et al., 2017), Bangladesh (Terao et al., 2015) and India (Abd Rani et al., 2011; Laha et al., 2014; Mandal et al., 2014, 2015). *B. gibsoni* infected dogs display serious clinical signs, including remittent fever, hemoglobinuria, progressive anemia,

marked splenomegaly and hepatomegaly, even sometimes resulting in mortality (Wozniak et al., 1997; Goo et al., 2008; Imre et al., 2013).

Transmission of *B. gibsoni* in fighting dogs was thought to be occurred by biting wounds, rather than by tick bites (Ikadai et al., 2004). However, there is a report of the tick infestation, which is the most dominant risk factor for *B. gibsoni* infection. The other routes of *B. gibsoni* transmission, includes vertical transmission, which is important in ordinary dogs in Japan (Fukumoto et al., 2005; Konishi et al., 2008). Because efficacious drugs and reliable rapid diagnostic kits yet to be developed (Irwin, 2009), there is need regarding further explore to the genome of *B. gibsoni* for potential vaccine and diagnostic antigens. However, the success of such approach will depend largely on identifying immunoprotective antigens that are stable against especially positive selection pressure (Fukumoto et al., 2003; Fukumoto et al., 2004).

5. Genetic manipulation of apicomplexan parasites

The initial reports on the successful establishment of transfection to the apicomplexan parasites were the description of transient and stable transfection methods for *Plasmodium* parasites and *Toxoplasma gondii* (Goonewardene et al., 1993; Kim et al., 1993; Soldati and Boothroyd, 1993; van Dijk et al., 1995). Subsequently, transient and stable transfection systems were developed for *Neospora caninum* (Howe and Sibley, 1997), *Eimeria tenella* (Kelleher and Tomley, 1998), *T. annulata* (Adamson et al., 2001), *B. bovis* (Suarez and McElwain, 2009), *Cryptosporidium parvum* (Li et al., 2009), *T. parva* (De Goeyse et al., 2015) and *B. ovata* (Hakimi et al., 2016).

In general, transient transfection method is designed to introduce and express exogenous gene, usually in the episomal plasmid, into a nucleated parasite in a non-stable manner. Thus, the introduced plasmid nucleic acid does not integrate into the genome of the target cells, and the transfected genes will not be replicated (Suarez et al., 2017). In contrast, stable transfection method is stably integrated exogenous gene into the parasite genome and expressed for an exogenous gene long period of time, or in some cases, it is maintained as extra-chromosomal replicating episomes (de Koning-Ward et al., 1999).

Recently, CRISPR/CAS9 system has been successfully applied to *P. falciparum*, *T. gondii* and *C. parvum* (Vinayak et al., 2015; Sidik et al., 2016; Kuang et al., 2017). The CRISPR is derived from clustered regularly-interspaced short palindromic repeats, which are part of an endonuclease from archaebacteria (Rath et al., 2015; Duan et al., 2016; Wright et al., 2016; Shmakov et al., 2017). The bacterial immune system provides RNA-mediated immunity against viruses and plasmids based on copying and specifically cleaving exogenous genetic materials (Karginov and Hannon, 2010; Barrangou, 2015). Overall, CRISPR/CAS9 is shown to be an efficient and specific tools for gene editing, and due to its high efficiency, CRISPR/CAS9 gradually replace the traditional gene method using homologous recombination mechanisms (Bortesi and Fischer, 2015; Price et al., 2015).

6. Genetic manipulation of *Babesia* parasites

The first study on the successful application of transient transfection was reported in *B. bovis*. The study described exogenous gene expression in *B. bovis* promoted by both *rhoptry associated protein-1* (*rap-1*) and *elongation factor-1 alpha*

(*ef-1α*) (Suarez et al., 2004, 2006). In the following work, the researcher described development and optimization of transfection method in *B. bovis* using both Nucleofection (Amaxa) and conventional electroporation (Gene Pulser II, BioRad). The optimal buffer (Plasmodium 88A6) and program (V-024) for Nucleofection based on free merozoites with plasmid containing luciferase gene as a reporter were determined (Suarez and McElwain, 2008).

The stable transfection system of *B. bovis* was established in 2009. A plasmid was designed to integrate GFP-BSD gene into the *B. bovis ef-1α* locus. *B. bovis*-iRBCs, biologically cloned Mo7 strain, were transfected with linear plasmid and were selected in cultures with blasticidin for 24 hours after transfection. Transfected parasite was selected for further analysis based on the GFP fluorescence in the presence of blasticidin (Suarez and McElwain, 2009).

In 2016, a stable transfection system of *B. ovata* was established using the *ef-1α* IG promoter for GFP expression and the actin 5' NR for expression of selectable marker hDHFR. The plasmid was designed to be integrated by double cross-over homologous recombination method into the *ef-1α* locus. Plasmid was transfected by electroporation into *in vitro* cultured *B. ovata* and facilitated by drug selection with WR99210 initiated 48 h after transfection. GFP-expressing parasites were observed by fluorescence microscopy after one-week cultivation with WR99210 (Hakimi et al., 2016).

Currently, *B. bigemina* transfection system was established using the identical method for the stable transfection of *B. bovis*. Stably transfected *B. bigemina* was obtained using a common transfection plasmid targeting the eGFP-BSD fusion gene into the *ef-1α* locus of *B. bigemina*. Sequencing, immunoblotting, Southern blotting

and immunofluorescence analysis of *B. bigemina*-iRBCs. Demonstrated that the eGFP-BSD gene was expressed and was stably integrated solely into the ef-1α locus of *B. bigemina* (Silva et al., 2018).

7. Aim of the present study

Genetic manipulation techniques, such as transfection, have been previously reported in many protozoan parasites. In *Babesia*, stable transfection systems have only been established for bovine *Babesia* parasites. The establishment of transfection system for *B. gibsoni* is considered to be urgent to improve our understanding on the basic biology of canine *Babesia* parasites and for control of canine babesiosis.

The objectives of the present study are as follows: (1) to establish a useful transient transfection system of *B. gibsoni*, towards a stable transfection method of *B. gibsoni*; (2) to identify and characterize of interchangeable cross-species functional promoters between *B. gibsoni* and *B. bovis*; (3) to establish a stable transfection system for *B. gibsoni*, towards a further genome editing of *B. gibsoni*.

Chapter 1

Establishment of a transient transfection system for genetic manipulation of *Babesia gibsoni*

1-1. Introduction

Babesia gibsoni is a tick-borne apicomplexan intraerythrocytic protozoan parasite which causes piroplasmosis of dogs (Liu et al., 2016). During the asexual phase of its life cycle occurring in the blood of the vertebrate host, *B. gibsoni* causes serious clinical signs such as anemia, hemoglobinuria, remittent fever, marked splenomegaly and hepatomegaly which may be fetal (Casapulla et al., 1998).

The difficulties in identifying *B. gibsoni* virulence factors and developing successful therapies have been attributed in part to the lack of genetic manipulation tools (Goo and Xuan, 2014). The development of these techniques have been reported in many protozoan parasites, such as *Plasmodium falciparum* (Ganesan et al., 2011), *Toxoplasma gondii* (Donald and Roos, 1993), *Trypanosoma cruzi* (Padmanabhan et al., 2014), *B. bovis* (Suarez and McElwain, 2009; Asada et al., 2012;), *Cryptosporidium parvum* (Vinayak et al., 2015), *Theileria annulata* (Adamson et al., 2001), *T. parva* (De Goeyse et al., 2015), *B. ovata* (Hakimi et al., 2016), and *B.*

bigemina (Silva et al., 2016). Application of transfection systems can lead to a better understanding of host-parasite interactions, the mechanisms underlying drug resistance and provides novel information for vaccine development and drug target discovery.

Elongation factor-1 alpha (*ef-1α*) is a constitutively expressed and abundant protein which is one of the key elements in eukaryotic protein translation (Suarez et al., 2006). Because of its high level of transcription, especially bidirectional promoter activity in *Plasmodium* and *Babesia* parasites (Vinkenoog et al., 1998; Hakimi et al., 2016), this promoter was widely used for gene expression in *Babesia* spp., such as *B. bigemina* and *B. bovis* (Suarez and McElwain, 2010; Silva et al., 2016).

1-2. Materials and methods

Parasite culture

B. gibsoni Oita strain (Sunaga et al., 2002) was cultured *in vitro* in 24-well culture plates (Thermo Fisher Scientific, USA) at 37°C in a humidified CO_2 (5%) and O_2 (5%) incubator (Bio-Lab, USA). The parasite was cultured in 10% canine red blood cells (RBCs) suspended in RPMI-1640 medium supplemented with 20% canine sera.

Animals

Beagle dogs (Nihon Nosan, Japan) were fed as donors for providing RBCs and sera.

Plasmid constructs

The schematic diagrams of plasmid pBS-ELA and pBS-EGA used in this study are shown in Fig. 2a and 3a. First, the firefly luciferase and green fluorescent protein (GFP) reporter genes were cloned into the pBluescript (pBS) back-bone plasmid, respectively, using the In-fusion HD Cloning Kit (Takara, Japan). Subsequently, the *B. gibsoni* 5′-intergenic (IG) region-B of the *ef-1α* gene (5′-*ef-1α*) and the 3′-untranslated region (UTR) of the *actin* gene (3′-*actin*) were amplified by polymerase chain reaction (PCR) and cloned into the upstream and downstream of the firefly luciferase and GFP reporter genes, respectively (Fig. 1). The primer pairs (Table 1) was designed based on *B. gibsoni* genome (unpublished data) and previous studies (Asada et al., 2012; Hakimi et al., 2016). The constructed plasmids were purified using Qiagen® Plasmid Midi Kit (Qiagen, Germany) according to the manufacturer's instructions, and the DNA sequence was confirmed by sequencing before transfection. The sequences of *B. gibsoni* 5′-*ef-1α* and 3′-*actin* were deposited into the GenBank database (accession numbers: KY171741 and KY171742).

Transfection of parasites

For transfection of *B. gibsoni*, the pBS-ELA and pBS-EGA plasmids were suspended in Tris–EDTA (TE, pH 8.0) buffer and parasite-infected red blood cells (iRBCs) at 8% parasitemia, respectively. The optimization programs for transfection were performed in 16-well Nucleocuvette strips (Fig. 2b and 2c) at a final volume of 20 µl. Parasite viability and transfection efficiency were assessed using a single Nucleocuvette (Fig. 2d, 2e, 2f, 3b and 3c) at a final volume of 100 µl. The plasmid-iRBCs mixtures were transfected using an Amaxa 4D Nucleofector™ device (Lonza, Germany) (De Goeyse et al., 2015; Vinayak et al., 2015) and immediately transferred into 1 ml of culture medium containing 10% fresh RBCs.

Luciferase assay and GFP-expressing parasites confirmation

The luciferase activity was measured at 24, 48 and 72 hours post transfection (hpt). The transfected iRBCs were spun down by centrifugation at 150 × *g* for 5 min and the supernatant was removed. Infected-RBCs pellet was suspended in 10 times volume of 0.8% NH_4Cl and incubated for 10 min at room temperature (RT) to rupture the erythrocytes. The cell pellet was then washed three times with PBS and spun down at 15,000 × *g* for 5 min. The final parasite pellet was resuspended in 30 µl of freshly-prepared Promega's 1X cell culture lysis reagent (Promega, USA) for the luciferase analysis as described previously (Suarez et al., 2004). The pellet was incubated for 15 min at RT for complete lysis and briefly centrifuged to remove any cell debris. Twenty microliters of cell lysate was mixed with 100 µl of the luciferase assay substrate (Promega, USA) and transferred in white 96-well plates. The mixture was incubated for 10 min at RT and the luminescence measured for 10 s integration interval by a GloMax®-Multi Detection System (Promega, USA). Hoechst 33342 (Sigma, USA) was used for confirming GFP-expressing parasites, while propidium iodide (PI) (Sigma, USA) (Pacheco-Lugo et al., 2017) and Hoechst 33342 were used for confirming parasite viability by fluorescence microscope (Keyence, USA).

Statistical analysis

Statistical analysis was performed by one-way ANOVA analysis of variance, followed by Tukey's multiple comparison test for program optimization, and Student's *t*-test were used for analysis of parasite viability and transfection efficiency (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

1-3. Results

Program optimization

The result of program optimization are shown in Fig. 2b and 2c. A preliminary test indicated that FA100 is a candidate program for transient transfection of *B. gibsoni* (Fig. 2b). Seven other programs close to FA100 were also analyzed. Program FA113 showed significantly higher luciferase activity than most of the other programs (Fig. 2c) (*$p < 0.05$, ***$p < 0.001$). Twenty micrograms of plasmid DNA was more efficient compared to 2, 5, 10 and 50 μg (Fig. 2d) (*$p < 0.05$, ***$p < 0.001$). At 10, 20 and 50 μg of plasmid DNA, buffer SF was more effective than buffer SG (Fig. 2d) (*$p < 0.05$, **$p < 0.01$).

Parasite viability

More than 87.7% of parasites remained viable 24 hpt in program FA113, which was significantly higher than the program V-024 (78.3%) regardless of the quantity of plasmid DNA used (Fig. 2e) (*$p < 0.05$, ***$p < 0.001$).

Time course of luciferase activity

Analysis of the time course of luciferase expression in *B. gibsoni* revealed high enzymatic activity at 24 hpt, followed by a rapid decline thereafter. However, parasite growth in post transfection culture increased exponentially with time. Transfection pulse induced parasite death was also observed in the current study, consistent with previous report in *B. bovis* (Suarez and McElwain, 2008).

Transfection efficiency

Transfection efficiency was determined by the percentage of GFP-expressing parasites at 24 hpt. Approximately 5% GFP-positive *B. gibsoni* was measured by fluorescence microscope. Statistical comparisons indicated that program FA113 showed significantly more transfection efficiency when compared to V-024 using 2 and 20 μg of plasmid DNA (Fig. 3b) (*$p < 0.05$, ***$p < 0.001$). The GFP-expressing parasites were also found at 48 and 72 hpt (Fig. 3c).

1-4. Discussion

Genetic manipulation techniques, such as transfection, have been previously reported in many protozoan parasites. In *Babesia*, stable transfection systems have only been established for bovine *Babesia* parasites. The establishment of transfection system for *B. gibsoni* is considered to be urgent to improve our understanding of the basic biology of canine *Babesia* parasites for a better control of canine babesiosis.

All previously established transfection systems for *Babesia* focused on bovine *Babesia* species, which were transfected using Gene Pulser Xcell™ Electroporation system (Bio-Rad, USA) and AMAXA Nucleofector™ 2b device (Lonza, Germany) (Suarez and McElwain, 2009; Hakimi et al., 2016; Silva et al., 2016). In this study, several programs of Gene Pulser Xcell™ Electroporation Systems (Bio-Rad, USA) and AMAXA Nucleofector™ 2b device (Lonza, Germany) were also attempted for transfection of *B. gibsoni*. However, parasite lysates did not show any luciferase activity, indicating failure in introduction of the plasmids into the parasite due to unsuitable buffer and transfection program. Therefore, the present method based on program FA113 and buffer SF of 4D Nucleofector™ may provide a more suitable transfection system for non-bovine *Babesia* parasites, such as *B. gibsoni*.

Nevertheless, stable transfection cannot be fully assessed based on the present study due to challenges such as low transfection efficiency and the need to overcome the rapid *in vitro* aging of canine erythrocytes (Lehtinen et al., 2008). Therefore, improve the transfection efficiency is urgently needed work. Additionally, to avoid the rapid aging of canine erythrocytes, sub-culturing every week by fresh RBCs is also needed. This finding is the first step towards the urgently needed stable transfection method for *B. gibsoni*, which may contribute to a better understanding of the biology of the parasite.

1-5. Summary

In summary, I present for the first time, the successful transient transfection of *B. gibsoni*. The plasmid containing the firefly luciferase reporter gene (pBS-ELA) was transfected into *B. gibsoni* by an AMAXA 4D Nucleofector™ device. Transfection using program FA113 and Lonza buffer SF showed the highest luciferase expression. Twenty micrograms of plasmid produced the highest relative transfection efficiency. The fluorescent protein-expressing parasites were determined by GFP-containing plasmid (pBS-EGA) at 48 and 72 hours post transfection. This finding is the first step towards a stable transfection method for *B. gibsoni*, which may contribute to a better understanding of the biology of the parasite and paves the way for the development of more efficient molecular-based subunit vaccines (or attenuated live vaccines) and discovery of novel drug targets. However, further efforts should be put into finding an effective selectable marker for developing a stable transfection system for *B. gibsoni*.

Table 1. List of primers used in the construction of pBS-ELA and pBS-EGA plasmid.

Plasmid	Primer	Sequence (5'→ 3')
pBS-ELA	5′-*ef-1α* (*Hind* III-F)	GACGGTATCGATAAGCTTCACTGTATAACGGATGAAGGT
	5′-*ef-1α* (*Hind* III-R)	GAATTCGATATCAAGCTTTTTGGTAAAGGTTGACGATA
	Luciferase (*Eco*R I-F)	AAGCTTGATATCGAATTCATGGAAGACGCCAAAAACAT
	Luciferase (*Eco*R I-R)	CCCGGGCTGCAGGAATTCTTACAATTTGGACTTTCCGCC
	3′-*actin* (*Pst* I-F)	TTGTAAGAATTCCTGCAGACGCAAAAAACAATCAACTACG
	3′-*actin* (*Pst* I-R)	GGATCCCCCGGGCTGCAGGAGCATCAACGGAATAGGGA
pBS-EGA	5′-*ef-1α* (*Hind* III-F)	GACGGTATCGATAAGCTTCACTGTATAACGGATGAAGGT
	5′-*ef-1α* (*Hind* III-R)	CACCATGATATCAAGCTTTTTGGTAAAGGTTGACGATA
	GFP (*Eco*R V-F)	ATCGATAAGCTTGATATCATGGTGAGCAAGGGCGA
	GFP (*Eco*R V-R)	CTGCAGGAATTCGATATCTTACTTGTACAGCTCGTCCATG
	3′-*actin* (*Bam*H I-F)	CTGCAGCCCGGGGGATCCACGCAAAAAACAATCAACTACG
	3′-*actin* (*Bam*H I-R)	TCTAGAACTAGTGGATCCGAGCATCAACGGAATAGGGA

Restriction enzyme sites are underlined.

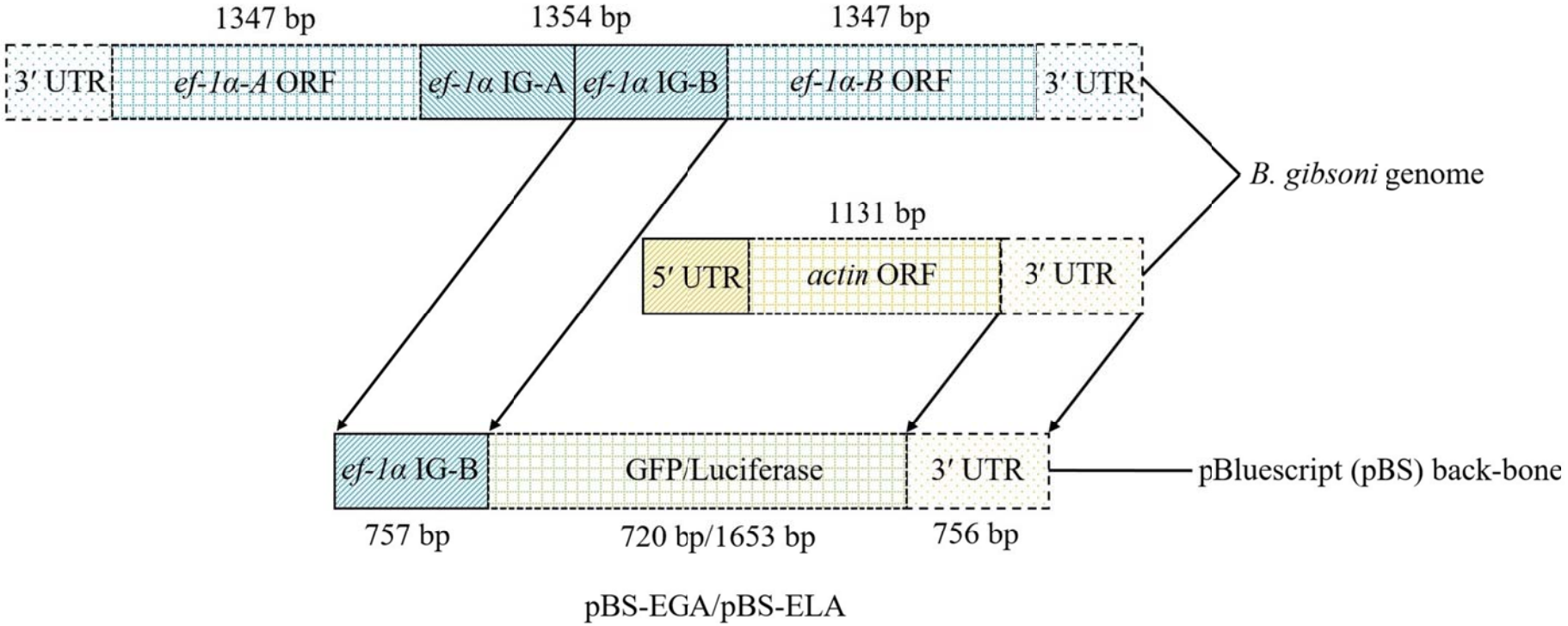

Fig. 1. Schematic diagram of the *Bg ef-1α* and *Bg actin* locus structure used in transient transfection of *B. gibsoni*.

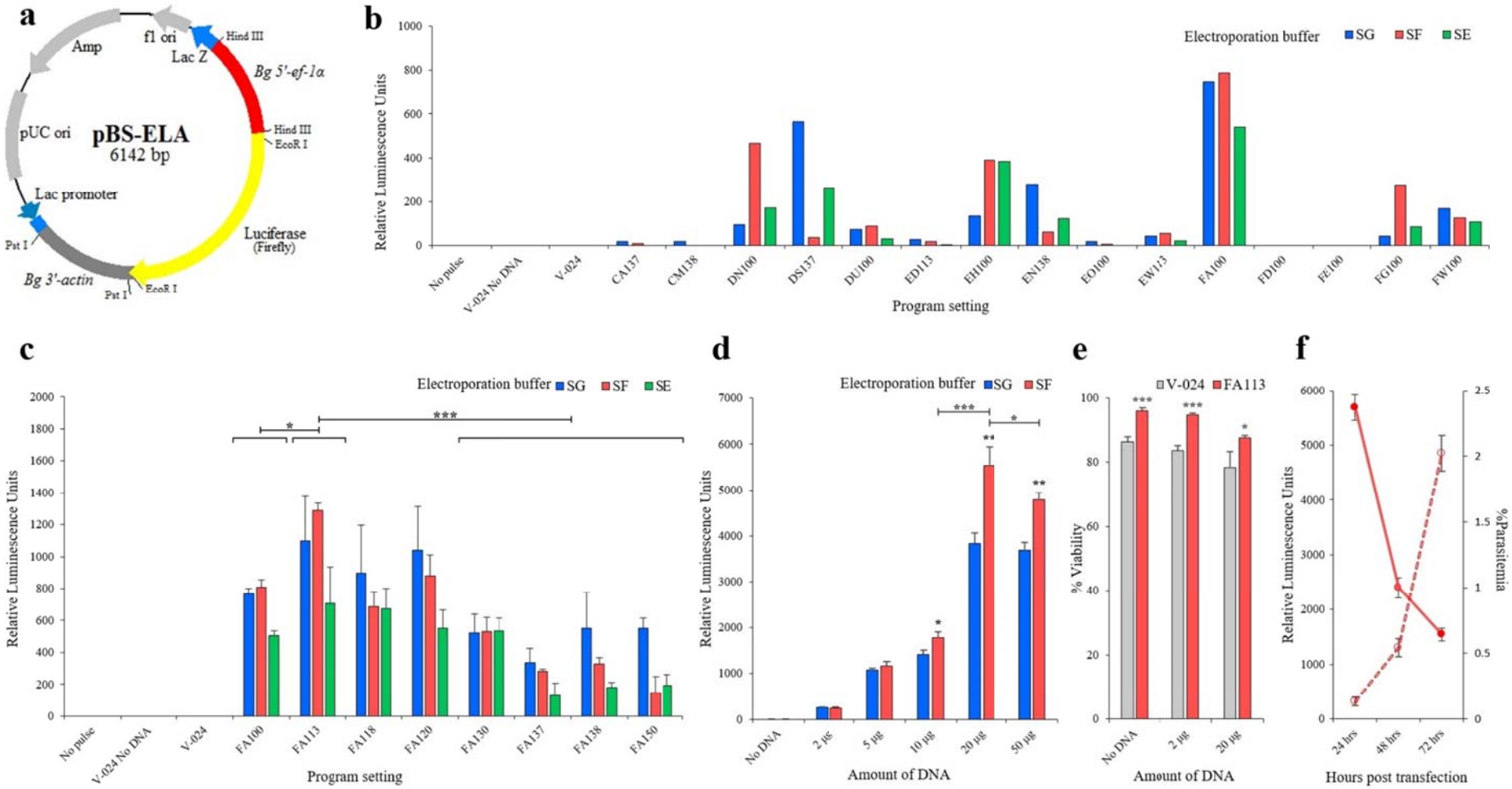
a
Amp
f1 ori
Lac Z
Hind III
Bg 5'-ef-1α
Hind III
EcoR I
pUC ori
pBS-ELA
6142 bp
Lac promoter
Luciferase
(Firefly)
Pst I
Bg 3'-actin
Pst I
EcoR I
b
Relative Luminescence Units
Electroporation buffer SG SF SE
No pulse
V-024 No DNA
V-024
CA137
CM138
DN100
DS137
DU100
ED113
EH100
EN138
EO100
EW113
FA100
FD100
FE100
FG100
FW100
Program setting
c
Relative Luminescence Units
Electroporation buffer SG SF SE
No pulse
V-024 No DNA
V-024
FA100
FA113
FA118
FA120
FA130
FA137
FA138
FA150
Program setting
d
Relative Luminescence Units
Electroporation buffer SG SF
No DNA
2 µg
5 µg
10 µg
20 µg
50 µg
Amount of DNA
e
% Viability
V-024 FA113
No DNA
2 µg
20 µg
Amount of DNA
f
Relative Luminescence Units
% Parasitemia
24 hrs
48 hrs
72 hrs
Hours post transfection

Fig. 2. a, The schematic diagram of plasmid pBS-ELA. b, The program optimization by 4D Nucleofection. 9 µl iRBCs (7.2×10^6 merozoites) and 9 µl Lonza buffers SG, SF or SE (4D Nucleofection) were combined with 2 µl (5 µg) pBS-ELA plasmid. Samples were transfected using V-024 (Nucleofection 2b) and various 4D Nucleofection program settings. Transfected iRBCs were mixed with fresh RBCs and luciferase activity was measured at 24 hours post transfection (hpt). c, Transfection was further optimized by comparing the best preliminary setting with additional pulse programs. Transfection was carried out as in b. d, Transfection by varying amounts of plasmid. 45 µl iRBCs (3.6×10^7 merozoites) and 45 µl buffer (SG or SF) were combined with 10 µl (2-50 µg) pBS-ELA plasmid. Samples were transfected by 4D Nucleofection FA113. Parasites were added to the cultures and luciferase activity was measured after 24 hpt. e, Parasite viability was determined by Hoechst 33342/PI double staining at 24 hpt. f, Time course of luciferase activity (full line) and parasitemia (dotted line) was measured at 24, 48 and 72 hpt. 45 µl iRBCs (3.6×10^7 merozoites) and 45 µl buffer SF were combined with 10 µl (20 µg) pBS-ELA plasmid. Samples were transfected by 4D Nucleofection FA113. The values were presented as means ± S.D. of three independent experiments. Statistical analysis was performed by one-way ANOVA analysis of variance, followed by Tukey's multiple comparison test in c, Student's *t*-test in d and e (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

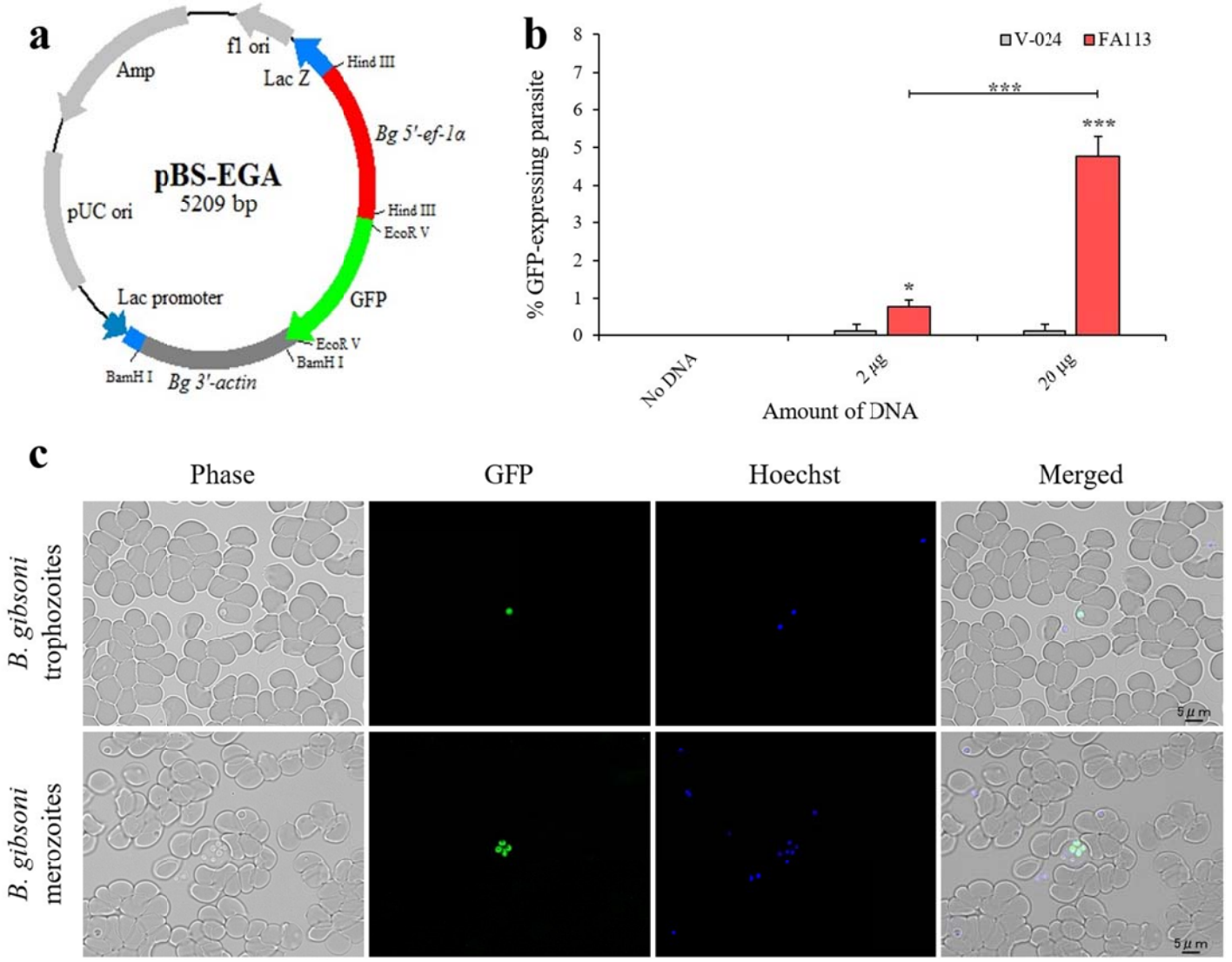
a
f1 ori
Hind III
Lac Z
Amp
Bg 5'-ef-1α
pBS-EGA
5209 bp
pUC ori
Hind III
EcoR V
Lac promoter
GFP
EcoR V
BamH I
BamH I
Bg 3'-actin
b
V-024
FA113
% GFP-expressing parasite
***
***
*
No DNA
2 μg
20 μg
Amount of DNA
c
Phase
GFP
Hoechst
Merged
B. gibsoni trophozoites
B. gibsoni merozoites
5 μm
5 μm

Fig. 3. a, The schematic diagram of plasmid pBS-EGA. b, Evaluation of transfection efficiency. GFP-expressing parasites were counted by fluorescence microscope at 24 hpt. The values were presented as means ± S.D. of three independent experiments. Statistical analysis was performed by Student's *t*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). c, Fluorescence microscope images of GFP-expressing parasites. Top panels show a transfected GFP-expressing *B. gibsoni* trophozoite detected at 48 hpt, bottom panels show a transfected GFP-expressing *B. gibsoni* merozoite detected at 72 hpt. Merged panels show overlap of GFP and Hoechst (parasite nuclei) fluorescence. The parasite nucleus was stained with Hoechst 33342.

Chapter 2

Identification and characterization of interchangeable cross-species functional promoters between *Babesia gibsoni* and *Babesia bovis*

2-1. Introduction

Babesiosis is a tick-transmitted, zoonotic disease caused by hematotropic parasites of the genus *Babesia* (Bonnet et al., 2009). *Babesia* parasites are some of the most ubiquitous and widespread blood parasites in the world (Homer et al., 2000). *Babesia gibsoni* and *B. bovis* are the main parasites responsible for canine and bovine babesiosis in terms of global parasite distribution, with considerable worldwide economic, and veterinary impact (Schnittger et al., 2012).

Transcriptional machinery of Apicomplexan parasites is unable to recognize viral promoters, such as the cytomegalovirus (CMV) and simian virus 40 (SV40) promoters (Meissner et al., 2001; Azevedo and Del Portillo, 2007). However, important elements for transcriptional control are interchangeable among some *Plasmodium* species (Crabb and Cowman, 1996; Mota et al., 2001). Recently, two distinct promoters with interchangeable homologous and heterologous promoter function were also identified in *B. bigemina* and *B. bovis* by *Elongation factor-1 alpha* (*ef-1α)* promoter (Silva et al., 2016). *Ef-1α* is a constitutively expressed and

abundant protein in eukaryotic protein translation (Suarez and McElwain, 2010). The promoter region of the *ef-1α* gene of *Plasmodium* and *Babesia* parasites has a high level of transcription and a bidirectional activity (Vinkenoog et al., 1998; Suarez et al., 2006).

Genetic manipulation is one of the ways through which novel therapeutic and preventing agents such as drugs and vaccines can be discovered against parasites. Perilously study reported that the challenges in developing effective treatment and vaccines against *B. gibsoni* may be partly attributed to lack of techniques for genetic manipulation of the parasite (Goo and Xuan, 2014). In Chapter I, I established a transient transfection of *B. gibsoni* by 5′-intergenic (IG) region-B of the *ef-1α* (*Bg 5′-ef-1α*) promoter. This being the only identified promoter for *B. gibsoni* transient transfection, there is a need for species specific or cross-species functional analysis of available promoters to advance our understanding of the molecular biology of *B. gibsoni.* Such knowledge will in turn contribute to the establishment of a stable transfection system in the future. This study identified and characterized interchangeable cross-species functional promoters between *B. gibsoni* and *B. bovis*.

2-2. Materials and methods

Parasites culture

The *B. gibsoni* Oita strain (Sunaga et al., 2002) and *B. bovis* Texas strain (Brayton et al., 2007) were used in this study. The *in vitro B. gibsoni* and *B. bovis* were cultured in 24-well culture plates (Thermo Fisher Scientific, USA) and grown at 37°C in humidified CO_2 (5%) and O_2 (5%) incubator (BIO-LABO, Japan). *B.*

gibsoni was cultured in 10% canine erythrocytes suspended in RPMI-1640 medium supplemented with 20% dog serum while *B. bovis* was cultured in 10% bovine erythrocytes suspended in GIT (Wako Pure Chemicals, Japan), and mediums were replaced every day.

Promoter prediction

Putative promoter regions used in this study were determined using the Promoter 2.0 Prediction Server (http://www.cbs.dtu.dk/services/Promoter/).

Plasmid constructs

The plasmid schematic diagrams used in this study are shown in Fig. 4a and 5a. First, the firefly luciferase and renilla luciferase genes were cloned into the pBluescript (pBS) back-bone plasmid, respectively, using the In-fusion HD Cloning Kit (Takara, Japan). Subsequently, the 3′-untranslated region (UTR) of the *B. bovis rap* gene (*3′-rap*) was cloned into the downstream of the firefly luciferase and renilla luciferase genes, respectively. Then, a total of 12 *Babesia* promoters, including *Bg 5′-ef-1α*-IG-B (*Bg 5′-ef-1α*), *Bb 5′-ef-1α*-IG-B (*Bb 5′-ef-1α*), *Bg 5′-actin*, *Bb 5′-actin*, *Bg 5′-thioredoxin peroxidase-1* (*tpx-1*), *Bb 5′-tpx-1*, *Bg 5′-heat shock protein 70* (*hsp70*), *Bb 5′-hsp70*, *Bg 5′-enolase*, *Bb 5′-enolase*, *Bg 5′-tubulin*, *Bb 5′-tubulin* were amplified from genomic DNA by PCR and cloned into the upstream of the firefly luciferase gene. Finally, to construct an internal control plasmid to normalize the promoter activity, *Bg 5′-ef-1α* and *Bb 5′-ef-1α* were cloned into the upstream of the renilla luciferase gene. All of the primer pairs (Table 2) were designed based on *B. gibsoni* genome (unpublished data), *B. bovis* genome and previous studies (Asada et al., 2012; Hakimi et al., 2016). The constructed plasmids were purified using

Qiagen® Plasmid Midi Kit (Qiagen, Germany) according to the manufacturer's instructions, and the DNA sequence was confirmed by sequencing before transfection.

Transfection of parasites

Babesia-infected red blood cells (iRBCs) were pretreated as described (Chapter 1). Transfections were conducted by introducing 20 µg of each firefly luciferase promoter plasmid together with 20 µg of renilla luciferase internal plasmid into *Babesia*-iRBCs. The *B. gibsoni*-iRBCs-plasmid and *B. bovis*-iRBCs-plasmid mixtures were electroporated using Amaxa 4D Nucleofector™ device (Lonza, Germany) and Amaxa Nucleofector™ 2b device (Lonza, Germany), respectively, and immediately transferred into 1 ml of culture medium containing 10% fresh RBCs.

Luciferase assay

The luciferase activity was measured by Dual-Glo® luciferase assay (Promega, USA) at 24 hours post transfection (hpt). The pellets were resuspended in 100 µl of freshly-prepared Promega′s 1X cell culture lysis reagent (Promega, USA). The pellets were incubated for 15 min at room temperature (RT) for complete lysis and briefly centrifuged to remove the cell debris. The firefly and renilla luciferase activity was measured for a 10 s integration interval using GloMax®-Multi Detection System (Promega, USA), successively. Readings from mock transfected parasites were subtracted from the firefly and renilla luciferase readings and the resulting values of firefly luciferase activity were normalized using the renilla luciferase activity for each sample. To evaluate promoter activities, three independent transfections were done for each promoter and each luciferase assay was performed in triplicate.

Bio-statistical analysis of promoter activities

The normalized luciferase activities were plotted using GraphPad Prism 6. Activity of each promoter candidates was performed by one-way ANOVA analysis of variance, followed by Dunnett's multiple comparison test with a promoter-less (control) plasmid. Student's *t*-test was used for the comparison between promoter candidates. Differences were statistically significant when $P<0.05$.

Statistical analysis

Statistical significance between promoter-less plasmid (No promoter) and other promoter candidates was performed by one-way ANOVA analysis of variance, followed by Dunnett's multiple comparison test. Student's *t*-test was used for the comparison between promoter candidates ($*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$).

2-3. Results

Promoter activities

A total of twelve promoters, consisting of 6 homologous and 6 heterologous promoters for *B. gibsoni* and *B. bovis*, were investigated. The result of homologous promoter function showed that *Bg 5′-actin* promoted a higher luciferase activity than *Bg 5′-ef-1α*.in *B. gibsoni* (Fig. 4). On the other hand, *Bg 5′-ef-1α* and *Bg 5′-actin* heterologous promoters resulted in significantly higher luciferase activity than *Bb 5′-ef-1α* homologous promoter in *B. bovis* (Fig. 5).

Cross-species function of promoters

Ten out of the 12 promoters had cross-species function and promoted significantly higher luciferase activity compared to the promoter-less control in both *B. gibsoni* and *B. bovis* (Figs. 4 and 5). The two tubulin promoters exhibited only homologous promoter function, with low but significant luciferase activity compared to the control (Figs 4 and 5).

Sequence homology among promoters

Sequence homology among the 12 promoters was 40.9-58.3% (Table 3). The *B. gibsoni* and *B. bovis* promoter sequences used in this study were deposited into the GenBank database (accession numbers: MF598081-MF598090).

2-4. Discussion

Previous transfection studies on *B. bovis* mainly focused on *Bb ef-1α* IG region and *Bb 5′-actin* promoters (Suarez et al., 2006; Asada et al., 2015), and in *B. gibsoni*, only *Bg 5′-ef-1α* promoter has been reported (Chapter 1). Of the 12 promoters identified in this study, *Bg 5′-actin* promoted higher luciferase activity than other 11 promoters, including *Bg 5′-ef-1α, Bb 5′-ef-1α* and *Bb 5′-actin* which were previously reported (Figs. 4 and 5). Interestingly, *Bg 5′-actin* promoter resulted in a higher luciferase activity in *B. bovis* than in *B. gibsoni*. This may be due either to *Bg 5′-actin* promoter having particular affinity for *B. bovis* transcription factors or to *B. bovis* lacking of proper regulatory signals for the regulation of the activity of *Bg 5′-actin* promoter. On the other hand, based on previous studies (Paparini et al., 2014; Hakimi et al., 2016), except for *Bg 5′-ef-1α* and *Bb 5′-ef-1α*, all the other promoters including *Bg 5′-actin* are unidirectional, and none of the determined promoters showed

conserved or similar structures (Table 3). Hence, *Bg 5′-actin* results may not be related to a bidirectional activity, and the identified cross-species function may not be due to sequence similarities in promoters. The observation that the heterologous promoter is more active than the homologous promoter was reported previously in *B. bovis* and *B. bigemina* (Silva et al., 2016).

Evaluation of different types of cross-species interchangeable promoters and their luciferase activities is useful in increasing the range of options of promoters for possible selection in transfection studies. The role of heterologous promoters in driving the expression of reporter genes in different species of apicomplexan parasites has been widely reported (Crabb and Cowman, 1996; Howe et al., 1997; Silva et al., 2016). However, some reports indicate that the heterologous promoters may be poorly or even not recognized at all by the transcriptional machinery (Azevedo and Del Portillo, 2007; Hakimi et al., 2016). The successful stable transfection of *B. bovis* (Suarez and McElwain, 2010) has created an opportunity for advanced genetic investigation of the parasite. Since the *B. gibsoni* genome is not yet published, I sought to mine for novel *B. bovis* interchangeable promoters that can be used for transfection of *B. gibsoni*. The current study identified 10 interchangeable cross-species functional promoters in both *B. gibsoni* and *B. bovis* (Figs. 4 and 5). Furthermore, my result also indicated that *Bb 3′-rap* terminator has cross-species function in *B. gibsoni*. Regrettably, due to the absence of data on *Bg 3′-rap* terminator, its interchangeable cross-species function could not be investigated in this study. The functional promoters and the terminator identified are expected to contribute towards the establishment of a stable transfection system for genetic manipulation of *B. gibsoni*. This result also indicates that heterologous promoter function widely exists between *B. gibsoni* and *B. bovis*. The promoters can recognize the transcription or

translation system mutually. These results will provide considerable flexibility in the future construction of plasmid vectors to be used for transfection systems in *Babesia* species.

2-5. Summary

In summary, this study identified and partially characterized 10 interchangeable cross-species functional promoters between *B. gibsoni* and *B. bovis*. *Bg 5′-ef-1α* and *Bg 5′-actin* heterologous promoters resulted in a significantly higher luciferase activity than *Bb 5′-ef-1α* homologous promoter in *B. bovis*. In particular, *Bg 5′-actin* was found to be the more active among the 12 tested promoter candidates. This study further indicates that heterologous promoter function widely exists between *B. gibsoni* and *B. bovis*. The data presented herein may be used to improve the already established transfection systems for *B. bovis*, and serve as a foundation for the future development of urgently needed gene editing and stable transfection systems for *B. gibsoni*.

Table 2. List of primers used to construct the plasmid of promoter evaluation.

Element	Primer	Sequence (5'→ 3')	Size (bp)
Promoter	*Bg 5'-ef-1α* (*Hind* III-F)	GACGGTATCGATAAGCTTCACTGTATAACGGATGAAGGT	757
	Bg 5'-ef-1α (*Hind* III-R)	GAATTCGATATCAAGCTTTTTGGTAAAGGTTGACGATA	
	Bb 5'-ef-1α (*Hind* III-F)	GACGGTATCGATAAGCTTCACGTAATAAATGAGATAAATAAGT	724
	Bb 5'-ef-1α (*Hind* III-R)	GAATTCGATATCAAGCTTTTTCGTAAAGTTGCAATAAATT	
	Bg 5'-actin (*Hind* III-F)	GACGGTATCGATAAGCTTCCAGTAAAAAGTGACTACCATA	1455
	Bg 5'-actin (*Hind* III-R)	GAATTCGATATCAAGCTTTTTGATAACGTAATAGCTCTGTA	
	Bb 5'-actin (*Hind* III-F)	GACGGTATCGATAAGCTTTGTACCAGGGATTG	1430
	Bb 5'-actin (*Hind* III-R)	GAATTCGATATCAAGCTTTTTGTCAAAAGCTATT	
	Bg 5'-tpx-1 (*Hind* III-F)	GACGGTATCGATAAGCTTACCGTGAGCGAAGGGGAC	974
	Bg 5'-tpx-1 (*Hind* III-R)	GAATTCGATATCAAGCTTTTATGTATATAGATATTTAG	
	Bb 5'-tpx-1 (*Hind* III-F)	GACGGTATCGATAAGCTTAACCAATTTACCAAACTCTGTA	723
	Bb 5'-tpx-1 (*Hind* III-R)	GAATTCGATATCAAGCTTTTGATGTTTAAATAATAATGCTT	
	Bg 5'-hsp70 (*Hind* III-F)	GACGGTATCGATAAGCTTCAGGCTAAAGAGAAGGGC	928
	Bg 5'-hsp70 (*Hind* III-R)	GAATTCGATATCAAGCTTTTTGATTCATCGACTACA	
	Bb 5'-hsp70 (*Hind* III-F)	GACGGTATCGATAAGCTTCCTACAAGACTTCTCAGCAACA	972
	Bb 5'-hsp70 (*Hind* III-R)	GAATTCGATATCAAGCTTTTGTTAAAATGAAGGCCAAG	
	Bg 5'-enolase (*Hind* III-F)	GACGGTATCGATAAGCTTCCCCTATTTATCACAGCG	952
	Bg 5'-enolase (*Hind* III-R)	GAATTCGATATCAAGCTTTGTCTAAAAAGCGTAAGCG	
	Bb 5'-enolase (*Hind* III-F)	GACGGTATCGATAAGCTTCTCCTTGACCTCTTTAGCCTTA	1036
	Bb 5'-enolase (*Hind* III-R)	GAATTCGATATCAAGCTTTTTGAGATAGAAGGAAGATGAAAA	
	Bg 5'-tubulin (*Hind* III-F)	GACGGTATCGATAAGCTTGAGTAAACGAGAATGGCA	1098
	Bg 5'-tubulin (*Hind* III-R)	GAATTCGATATCAAGCTTCCTGTATAATCATGAAAT	
	Bb 5'-tubulin (*Hind* III-F)	GACGGTATCGATAAGCTTAGCAGGCTTACTCGCAGGGT	1383
	Bb 5'-tubulin (*Hind* III-R)	GAATTCGATATCAAGCTTCGTCGCATGTCGGAATGAAG	
Reporter	Firefly luciferase (*Eco*R I-F)	AAGCTTGATATCGAATTCATGGAAGACGCCAAAAACAT	1653
	Firefly luciferase (*Eco*R I-R)	CCCGGGCTGCAGGAATTCTTACAATTTGGACTTTCCGCC	
	Renilla luciferase (*Eco*R I-F)	AAGCTTGATATCGAATTCATGACTTCGAAAGTTTATGATCCA	936
	Renilla luciferase (*Eco*R I-R)	CCCGGGCTGCAGGAATTCTTATTGTTCATTTTTGAGAACTCG	
Terminator	*Bb 3'-rap* (*Pst* I-F)	TAAGAATTCCTGCAGGATGAGATGCGTTTATAATGGC	1283
	Bb 3'-rap (*Pst* I-R)	GGATCCCCCGGGCTGCAGCCTACGAACGATATGTCAAAGAG	

Restriction enzyme sites are underlined.

Table 3. Comparison of the sequences of the promoters used in this study.

Promoter		1	2	3	4	5	6	7	8	9	10	11	12
Bg 5'-ef-1α	1		0.496	0.469	0.493	0.465	0.441	0.469	0.452	0.473	0.480	0.457	0.476
Bb 5'-ef-1α	2	0.859		0.485	0.459	0.473	0.471	0.494	0.496	0.434	0.485	0.472	0.477
Bg 5'-actin	3	1.032	0.906		0.440	0.472	0.447	0.452	0.476	0.487	0.457	0.447	0.462
Bb 5'-actin	4	0.881	0.988	1.092		0.456	0.452	0.450	0.455	0.454	0.465	0.478	0.463
Bg 5'-tpx-1	5	1.001	0.932	0.942	1.006		**0.583**	0.433	0.467	0.428	0.452	0.439	0.482
Bb 5'-tpx-1	6	1.053	0.952	1.045	1.051	0.626		0.466	0.496	0.460	0.459	0.442	0.474
Bg 5'-hsp70	7	0.969	0.896	1.053	1.030	1.123	0.966		0.431	0.416	0.466	0.459	0.486
Bb 5'-hsp70	8	1.060	0.861	0.953	1.030	0.966	0.884	1.114		**0.409**	0.446	0.433	0.505
Bg 5'-enolase	9	0.942	1.136	0.902	1.029	1.112	1.016	1.254	1.234		0.571	0.438	0.470
Bb 5'-enolase	10	0.912	0.904	1.007	0.978	1.050	0.990	0.967	1.059	0.646		0.451	0.448
Bg 5'-tubulin	11	1.016	0.952	1.066	0.924	1.082	1.067	1.030	1.142	1.103	1.047		0.498
Bb 5'-tubulin	12	0.956	0.929	0.985	1.005	0.906	0.957	0.913	0.854	0.971	1.054	0.857	

The numbers in the upper right half and lower left half in the table, represent the ratio of sequence identities and divergences between promoter regions, respectively.

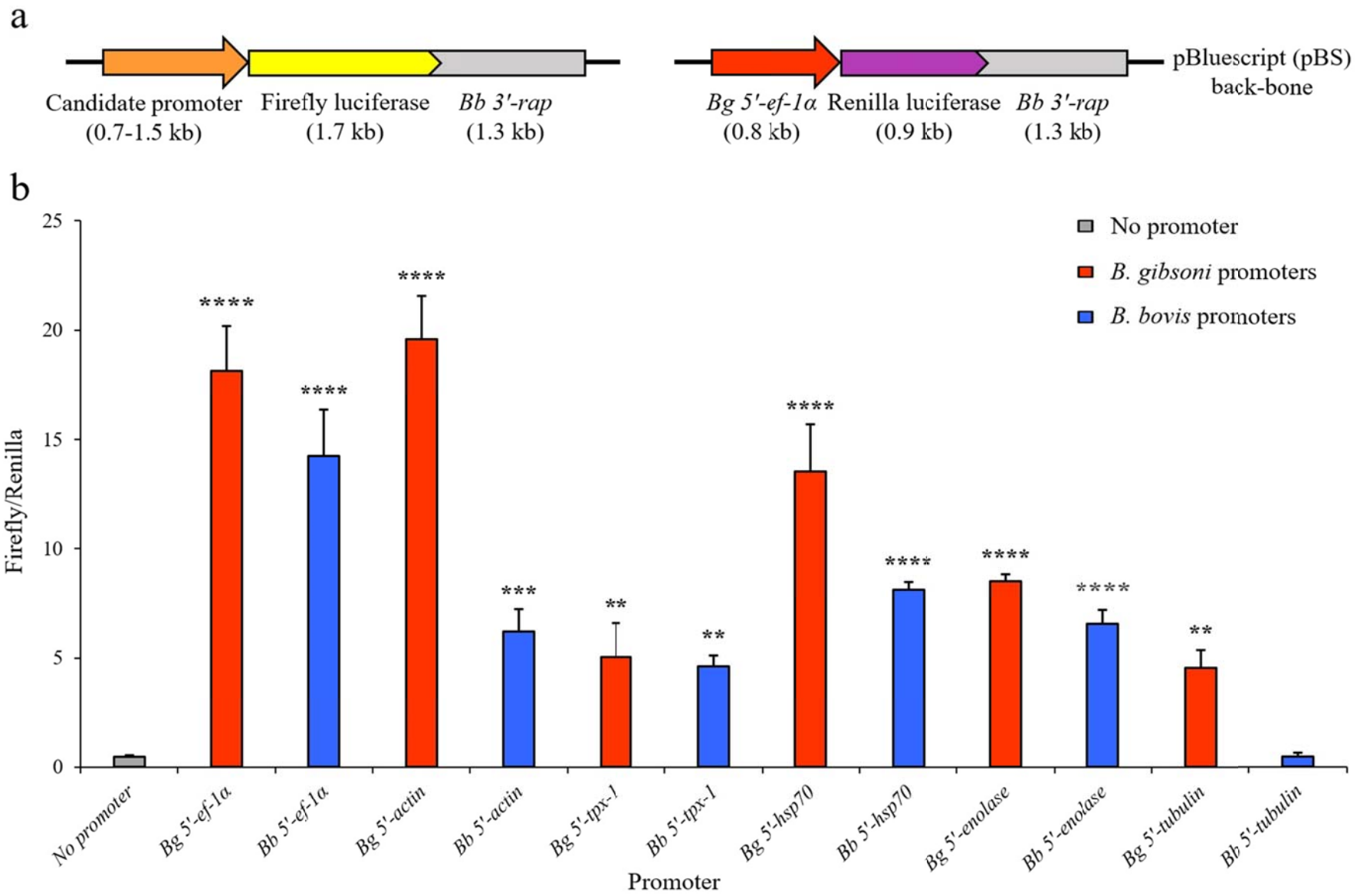
a
Candidate promoter
(0.7-1.5 kb)
Firefly luciferase
(1.7 kb)
Bb 3'-rap
(1.3 kb)
Bg 5'-ef-1α
(0.8 kb)
Renilla luciferase
(0.9 kb)
Bb 3'-rap
(1.3 kb)
pBluescript (pBS)
back-bone
b
No promoter
B. gibsoni promoters
B. bovis promoters
Firefly/Renilla
25
20
15
10
5
0
****
****
****
***
**
**
****
****
****
**
No promoter
Bg 5'-ef-1α
Bb 5'-ef-1α
Bg 5'-actin
Bb 5'-actin
Bg 5'-tpx-1
Bb 5'-tpx-1
Bg 5'-hsp70
Bb 5'-hsp70
Bg 5'-enolase
Bb 5'-enolase
Bg 5'-tubulin
Bb 5'-tubulin
Promoter

Fig. 4. a, Schematic diagram of plasmid construct to evaluate the promoter activity in *B. gibsoni*, and a Renilla luciferase-expressing plasmid for normalization. b, Comparison of luciferase activity in lysates of *B. gibsoni* transfected with different constructs at 24 hours post transfection (hpt). A promoter-less plasmid was used as a negative control. The values were presented as means ± S.D. of three independent experiments. Statistical significance between promoter-less plasmid (No promoter) and other promoter candidates was performed by one-way ANOVA analysis of variance, followed by Dunnett's multiple comparison test (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$).

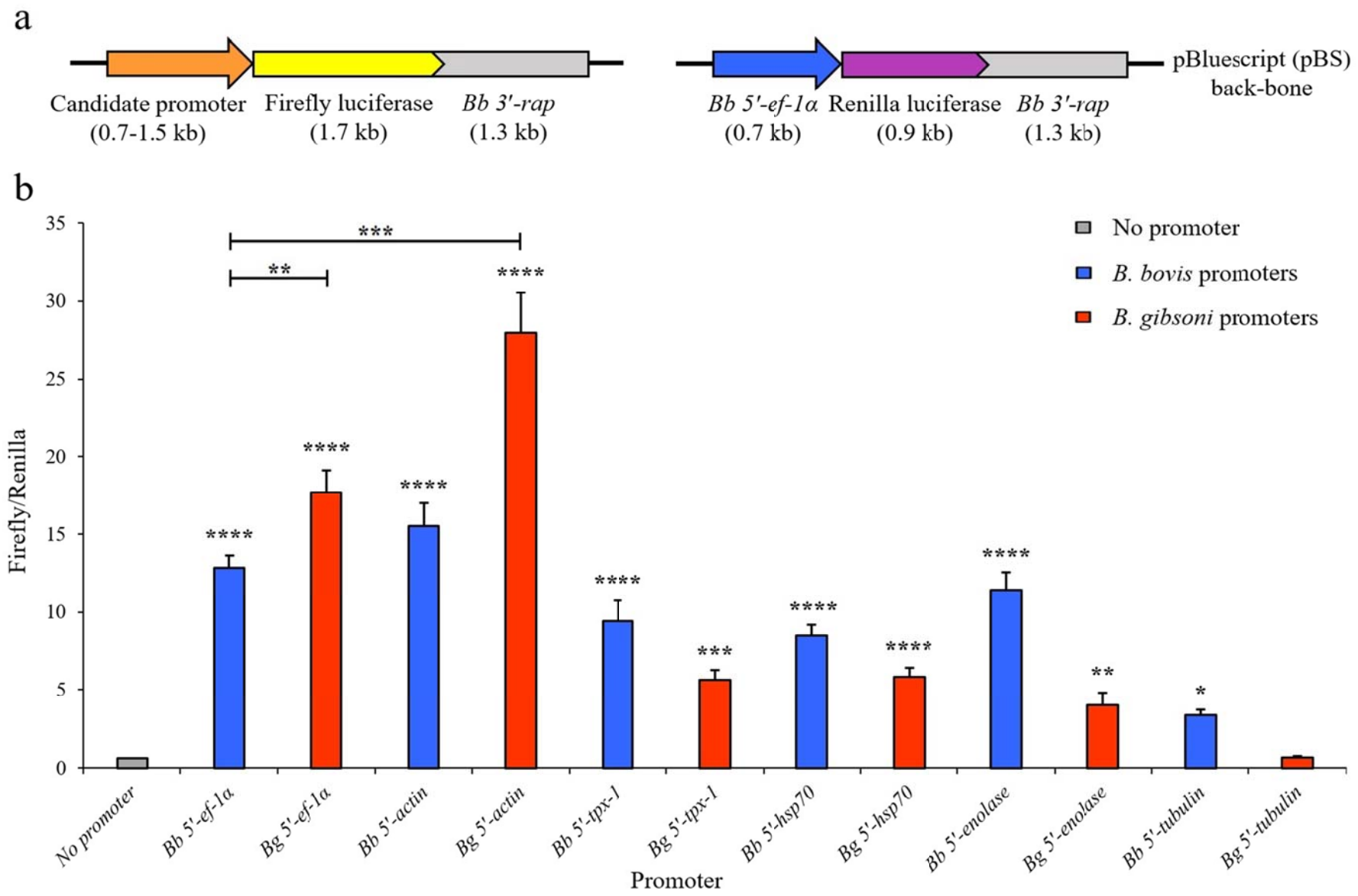
a
Candidate promoter
(0.7-1.5 kb)
Firefly luciferase
(1.7 kb)
Bb 3'-rap
(1.3 kb)
Bb 5'-ef-1α
(0.7 kb)
Renilla luciferase
(0.9 kb)
Bb 3'-rap
(1.3 kb)
pBluescript (pBS)
back-bone
b
No promoter
B. bovis promoters
B. gibsoni promoters
Firefly/Renilla
Promoter
No promoter
Bb 5'-ef-1α
Bg 5'-ef-1α
Bb 5'-actin
Bg 5'-actin
Bb 5'-tpx-1
Bg 5'-tpx-1
Bb 5'-hsp70
Bg 5'-hsp70
Bb 5'-enolase
Bg 5'-enolase
Bb 5'-tubulin
Bg 5'-tubulin

Fig. 5. a, Schematic diagram of plasmid construct to evaluate the promoter activity in *B. bovis*, and a Renilla luciferase-expressing plasmid for normalization. b, Comparison of luciferase activity in lysates of *B. bovis* transfected with different constructs at 24 hours post transfection (hpt). A promoter-less plasmid was used as a negative control. The values were presented as means ± S.D. of three independent experiments. Statistical significance between promoter-less plasmid (No promoter) and other promoter candidates was performed by one-way ANOVA analysis of variance, followed by Dunnett's multiple comparison test. Student's *t*-test was used for the comparison between promoter candidates (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$).

Chapter 3

Establishment of a stable transfection system for genetic manipulation of *Babesia gibsoni*

3-1. Introduction

Transient and stable transfection systems have been established for several apicomplexan parasites, such as *Plasmodium falciparum* (Ganesan et al., 2011), *Toxoplasma gondii* (Donald and Roos, 1993), *Cryptosporidium parvum* (Vinayak et al., 2015), *Theileria annulata* (Adamson et al., 2001) and *T. parva* (De Goeyse et al., 2015). Among *Babesia* species, transient and stable transfection systems have been reported for *B. bovis* (Suarez and McElwain, 2009), *B. ovata* (Hakimi et al., 2016) and *B. bigemina* (Silva et al., 2016). For *B. gibsoni*, I have described transient transfection systems (Chapter 1, Chapter 2). *B. gibsoni elongation factor-1 alpha* (*Bg 5′-ef-1α*) promoter, Program FA113 of AMAXA 4D Nucleofector™ and Lonza buffer SF successfully supported the expression of reporter genes (Chapter 1). In addition, among the 12 promoter candidates tested, *Bg 5′-actin* was found to be the most active promoter (Chapter 2). Similar to *B. bovis* (Suarez and McElwain, 2010) the development of a stable transfection system for *B. gibsoni* parasites requires a drug selection system and an integration target. The WR99210/human dihydrofolate reductase gene (*hdhfr*) selection system and double cross-over homologous

recombination locus have previously been successfully used for *B. bovis* (Asada et al., 2012) and *B. ovata* (Hakimi et al., 2016) stable transfection.

In this study, in order to establish *B. gibsoni* stable transfection, I investigated whether stable transfection of GFP-expressing *B. gibsoni* could be achieved using *hdhfr* as a selectable marker under the control of the *Bg 5'-ef-1α* (IG-B) and *Bg 5'-actin* promoters, and *ef-1α* locus as the integration target.

3-2. Materials and methods

Parasite culture

In this study, *B. gibsoni* Oita strain (Sunaga et al., 2002) was cultured *in vitro* in 24-well culture plates (Thermo Fisher Scientific, USA) at 37 °C in humidified CO_2 (5%) and O_2 (5%) incubator (BIO-LABO, Japan). The parasite was cultured in 10% canine erythrocytes suspended in RPMI-1640 medium supplemented with 20% canine serum.

Evaluation of *B. gibsoni* sensitivity to WR99210

B. gibsoni was cultured *in vitro* in 96-well culture plates with 100 µl RPMI-1640 medium containing 10% canine erythrocytes supplemented with 20% dog serum and different concentrations of WR99210 (0.1, 0.5, 1, 5, 10 and 100 nM). For each drug concentration, parasites were cultured in triplicate wells and the culture medium was replaced daily. Parasitemia was calculated on day 3 by examining 3,000 RBCs of a prepared thin blood smear stained with Giemsa solution.

Plasmid constructs

The schematic diagram of the plasmid used in this study (pBS-EGRADE) is shown in Fig. 7a. The reporter gene and drug selection gene cassettes were separated in order to drive *gfp* and *hdhfr* with *Bg 5′-ef-1α* (IG-B) and *Bg 5′-actin* promoters, respectively. *Bg 5′-ef-1α* (IG-B) and *Bg 3′-ef-1α* were used as recombination sites cloned into the upstream and downstream of the *gfp* and *hdhfr* genes, respectively. All the PCR primer pairs used for plasmid construction are listed in Table 1 and restriction sites are underlined. The constructed plasmid was purified using Qiagen® Plasmid Maxi Kit (Qiagen, Germany) according to the manufacturer's instructions, and was confirmed by sequencing before transfection. The sequence of pBS-EGRADE plasmid was deposited in the GenBank database under the accession number MG913246.

Transfection of parasites

B. gibsoni-infected red blood cells (iRBCs) were pre-treated as previously described (Chapter 1). Transfection was conducted using 20 μg of linearized pBS-EGRADE plasmid. The plasmid-iRBCs mixtures were transfected using Lonza buffer SF and program FA113 of Amaxa 4D Nucleofector™ device (Lonza, Germany) and immediately transferred into a preheated culture containing 10% fresh RBCs. To avoid the rapid *in vitro* aging of canine erythrocytes, transfected parasites were sub-cultured every week and supplemented with fresh RBCs. To select GFP-expressing transgenic parasites, 10 nM WR99210 was added to the culture medium two days after transfection. After 4 weeks of drug selection, the parasite population was cloned in a 96-well culture plate using limiting dilution as previously described (Asada et al., 2012).

PCR characterization of GFP-expressing *B. gibsoni*

Three sets of primers (Table 4) were used to confirm the integration of pBS-EGRADE into *B. gibsoni ef-1α* locus. Primer pair Integ-F and GFP-R was used to amplify a 1.6 kb DNA fragment to confirm the 5′ recombination. Primer pair hDHFR-F and Integ-R was used to amplify a 2.0 kb DNA fragment to examine the 3′ recombination whereas primer pair GFP-F and hDHFR-R was used to amplify a 4.1 kb DNA fragment to detect the insertion region. The DNA fragments amplified were confirmed by sequencing.

Southern blot analysis

Two micrograms of genomic DNA from wild type (WT) and genome integrated (GI) *B. gibsoni* were digested overnight with 20 units of *Sca* I and *Sph* I. The digestion products were separated by agarose gel electrophoresis, transferred onto Hybond N^+ membrane (GE Healthcare, UK) then hybridized with labeled probes using an AlkPhos Direct Kit (GE Healthcare, UK) according to the manufacturer′s instructions. Two probes corresponding to the complete open reading frame (ORF) of *gfp* and the 0.4 kb length of *Bg 3′-ef-1α* fragment, respectively, were used. The primer pairs used to amplify the probes are listed in Table 1. Probe signal was detected using a CDP-star detection reagent (GE Healthcare, UK).

Growth curves

WT and GI parasites were continuously cultured from approximately 0.5% parasitemia by sub-culturing every 3 days for two generations. Parasitemia were monitored daily by examining 3000 RBCs with Giemsa staining.

3-3. Results

B. gibsoni **sensitivity to WR99210**

WR99210 successfully inhibited the growth of *B. gibsoni in vitro* at a nanomolar concentration (Fig. 6). The calculated IC_{50} was 1.1 nM, and 10 nM WR99210 completely inhibited the growth of *B. gibsoni*. Thus, 10 nM WR99210 was used for the selection of transfected parasites.

Establishment of stable GFP expression in ***B. gibsoni***

GFP-expressing parasites emerged as early as two weeks after drug selection with 10 nM WR99210. The parasite population was cloned by limiting dilution and consistently expressed GFP for more than 3 months without drug pressure (Fig. 7b). After obtaining parasite clonal lines, the correct integration of pBS-EGRADE into the *ef-1α* locus was confirmed by the results of both PCR and Southern blot analysis. The PCR-1, -2 and -3 primer pairs successfully amplified 1.6, 2.0 and 4.1 kb DNA fragments, respectively, using DNA template from one clonal line named GI parasite (Fig. 8a) and the amplified DNA fragments were validated by sequencing. The sequences of the above DNA fragments were deposited in the GenBank database under the accession numbers MH087225-MH087227. No amplicons were obtained with DNA template from the WT parasite. In Southern blot analysis, both *gfp* and *3′-ef-1α* probes detected a single 5.5 kb band for GI parasite, while the *3′-ef-1α* probe detected a single 2.1 kb band, and the *gfp* probe did not detect any band for the WT parasite (Fig. 8b). In addition, the growth curves of WT and GI parasites showed high similarity (Fig. 9).

3-4. Discussion

Transfection systems improve our understanding of the molecular biology of parasites and pave the way for genetic manipulation (Suarez et al., 2017). The application of transfection systems can also lead to a better understanding of the mechanisms underlying drug resistance, host-parasite interactions, and provide novel information for vaccine development and drug target discovery (Suarez and Noh, 2011). Currently, there is a lack of techniques for the genetic manipulation of *B. gibsoni*. In order to fill this gap, I describe herein the development of a stable transfection system for *B. gibsoni*.

In this study, I employed a WR99210/*hdhfr* selection system for *B. gibsoni stable transfection.* The IC_{50} of WR99210 against *B. gibsoni* was 1.1 nM (Fig. 6), which is similar to *B. bovis* (1 nM) (Asada et al., 2012) and almost twice that of the one reported for *B. ovata* (0.56 nM) (Hakimi et al., 2016). The transfected parasite selected with WR99210/*hdhfr* emerged as early as two weeks after adding the drug, indicating the suitability of this selection system for *stable transfection of B. gibsoni. Babesia bovis 3′-rap* was successfully used as terminator in this study (Fig. 7a). This result is consistent with my previous work (Chapter 2), confirming that *Bb 3′-rap* heterologous terminator is fully functional in *B. gibsoni*. These findings provide considerable flexibility in the construction of plasmid vectors to be used for transfection systems in *Babesia* species. The cloned GI parasite stably expressed GFP (Fig. 7b) and *PCR amplicons* (Fig. 8a) *and Southern blot analyses* (Fig. 8b) *indicated that* pBS-EGRADE was integrate*d into B. gibsoni genome* by homologous recombination *as expected.* In addition, the growth of GI parasite was comparable with that of the WT parasite (Fig. 9). These results indicate that the genetic manipulations in this study did not affect the growth of parasite *in vitro*.

The proliferation of *Babesia* organisms in the vectors is an essential part of their survival. However, the detailed life-cycle of the parasite in ticks, including information about the timing of migration, remains unknown (Maeda et al., 2016). *Haemaphysalis longicornis*, a vector for *B. gibsoni* (Iwakami et al., 2014), is widely used as a model tick to study pathophysiology in tick infestation (Islam et al., 2009). Therefore, transfected *B. gibsoni* and *H. longicornis* could be used for developing tick-*Babesia* experimental models for clarifying the kinetics of the tick stage of canine *Babesia* parasites. A tick-*Babesia* interactions model may contribute to a better understanding of tick transmission as well as the way *Babesia* species interact with the ticks.

All previously established transfection systems for *Babesia* focused on bovine *Babesia* species, which were transfected using Gene Pulser Xcell™ Electroporation system (Bio-Rad, USA) and AMAXA Nucleofector™ 2b device (Lonza, Germany) (Suarez and McElwain, 2009; Hakimi et al., 2016; Silva et al., 2016). However, these transfection systems were not effective for *B. gibsoni* (Chapter 1). Therefore, the present method based on 4D Nucleofector™ may provide a more suitable transfection system for non-bovine *Babesia* parasites, such as *B. gibsoni*. The rapid *in vitro* aging of canine erythrocytes (Lehtinen et al., 2008) may play an important role in restricting a successful transfection. Therefore, to avoid the rapid aging of canine erythrocytes, I strongly suggest sub-culturing every week by fresh RBCs after transfection. A host-*Babesia* infection model may be easier to achieve using canine *Babesia* rather than bovine *Babesia* because using dogs for animal experiments is more feasible than using cattle. The urgently needed genome edited host-*Babesia* infection model may help us monitor transmission *in vivo*, investigate mechanisms of infection and

immunity, and also improve the development of novel strategies for controlling babesiosis.

3-5. Summary

In summary, I established a stable transfection system for *B. gibsoni* and successfully integrated exogenous genes into the *B. gibsoni* genome. The establishment of this system is critical to fulfill genome editing, which may contribute to determining gene function, discovery of novel drug targets and evaluation of the interactions between the parasite and the host. This finding will also facilitate functional analysis of *Babesia* genomes using genetic manipulation and will serve as a foundation for the development of tick-*Babesia* and host-*Babesia* infection models.

Table 4. List of primers used in this study

Primer	Sequence (5'–3')[a]
Bg 5′-ef-1α-F (*Hind* III)	GACGGTATCGATAAGCTTCACTGTATAACGGATGAAGGT
Bg 5′-ef-1α-R (*Hind* III)	CACCATGATATCAAGCTTTTTGGTAAAGGTTGACGATA
GFP-F (*Eco*R V)	ATCGATAAGCTTGATATCATGGTGAGCAAGGGCGA
GFP-R (*Eco*R V)	CTGCAGGAATTCGATATCTTACTTGTACAGCTCGTCCATG
Bb 3′-rap-F (*Sma* I)	GAATTCCTGCAGCCCGGGGATGAGATGCGTTTATAATGGC
Bb 3′-rap-R (*Sma* I)	ACTAGTGGATCCCCCGGGCCTACGAACGATATGTCAAAGAG
Bg 5′-actin-F (*Hind* III)	GACGGTATCGATAAGCTTCCAGTAAAAAGTGACTACCATA
Bg 5′-actin-R (*Hind* III)	AACCATGATATCAAGCTTTTTGATAACGTAATAGCTCTGTA
hDHFR-F (*Eco*R V)	ATCGATAAGCTTGATATCATGGTTGGTTCGCTAAAC
hDHFR-R (*Eco*R V)	CTGCAGGAATTCGATATCTTAATCATTCTTCTCATATACTTC
Bg 3′*-ef-1α*-F (*Sma* I)	GAATTCCTGCAGCCCGGGAGCTGATTATTTCGTGTTAACT
Bg 3′*-ef-1α*-R (*Sma* I)	ACTAGTGGATCCCCCGGGGATTGGTAGTATTTGTCGTCAT
EGR-F (*Sal* I)	CCCCCCCTCGAGGTCGACCACTGTATAACGGATGAAGGT
EGR-R (*Sal* I)	CTTATCGATACCGTCGACCCTACGAACGATATGTCAAAGAG
Integ-F	TAGCAGCCAAGCGAGATA
Integ-R	CAACTTAGATTGATCGGTG
Probe-GFP-F	ATGGTGAGCAAGGGCGA
Probe-GFP-R	TTACTTGTACAGCTCGTCCATG
Probe-3′*-ef-1α*-F	ATCCCCTGTCTCAATGG
Probe-3′*-ef-1α*-R	GATTGGTAGTATTTGTCGTCA

[a]Restriction enzyme sites are underlined

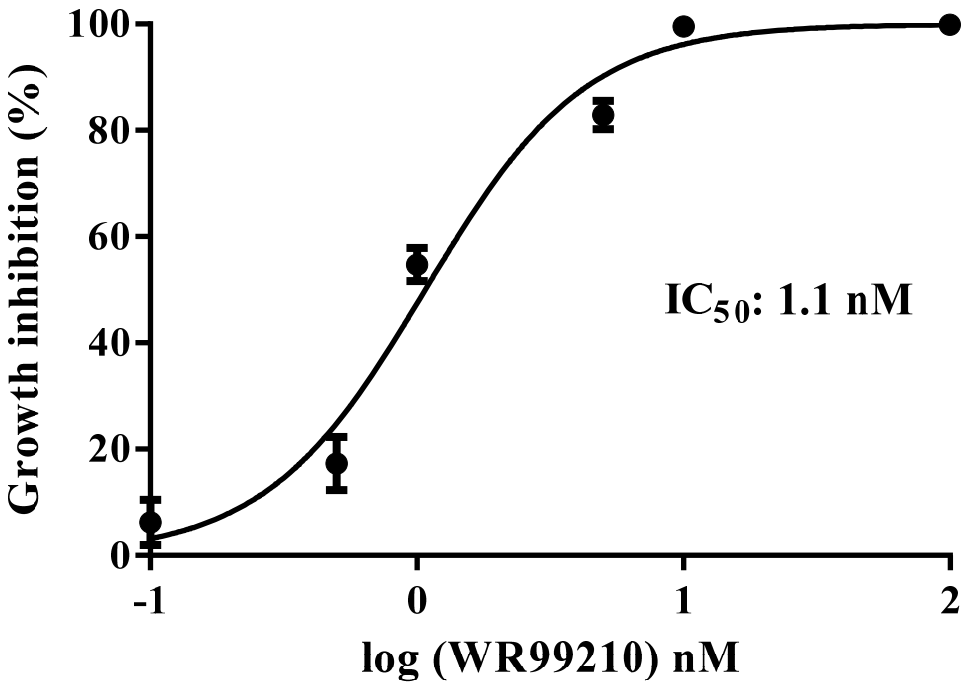

Fig. 6. *B. gibsoni* sensitivity to WR99210. All data are expressed as means ± SD of triplicate cultures.

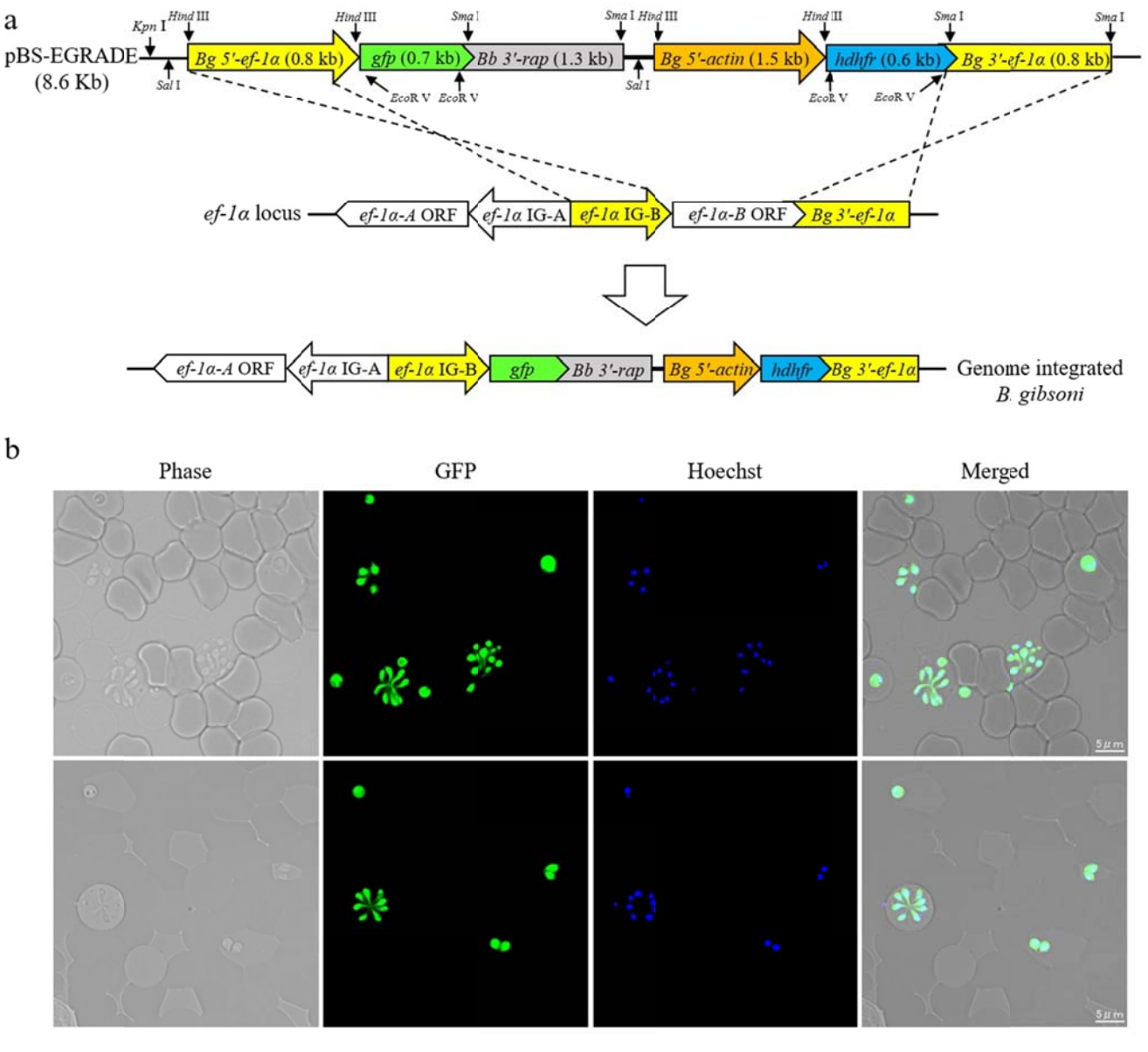
a
pBS-EGRADE
(8.6 Kb)
Kpn I
Hind III
Sal I
Bg 5′-ef-1α (0.8 kb)
Hind III
Sma I
gfp (0.7 kb)
Bb 3′-rap (1.3 kb)
EcoR V
EcoR V
Sma I
Hind III
Sal I
Bg 5′-actin (1.5 kb)
Hind III
EcoR V
hdhfr (0.6 kb)
Sma I
EcoR V
Bg 3′-ef-1α (0.8 kb)
Sma I
ef-1α locus
ef-1α-A ORF
ef-1α IG-A
ef-1α IG-B
ef-1α-B ORF
Bg 3′-ef-1α
ef-1α-A ORF
ef-1α IG-A
ef-1α IG-B
gfp
Bb 3′-rap
Bg 5′-actin
hdhfr
Bg 3′-ef-1α
Genome integrated
B. gibsoni
b
Phase
GFP
Hoechst
Merged
5 μm
5 μm

Fig. 7. Schematic diagram of GFP-expressing plasmid (pBS-EGRADE) construct and fluorescence microscopy images of stably expressing GFP *B. gibsoni*. a, Plasmid construct of pBS-EGRADE showing the recombination sites for integration into *ef-1α* locus by double cross-over homologous recombination. The restriction site for linearization (*Kpn* I) is shown. b, Fluorescence microscopy images of stable GFP-expressing *B. gibsoni*. Merged panel shows overlap of GFP and Hoechst (parasite nuclei) fluorescence. The parasite nucleus was stained with Hoechst 33342

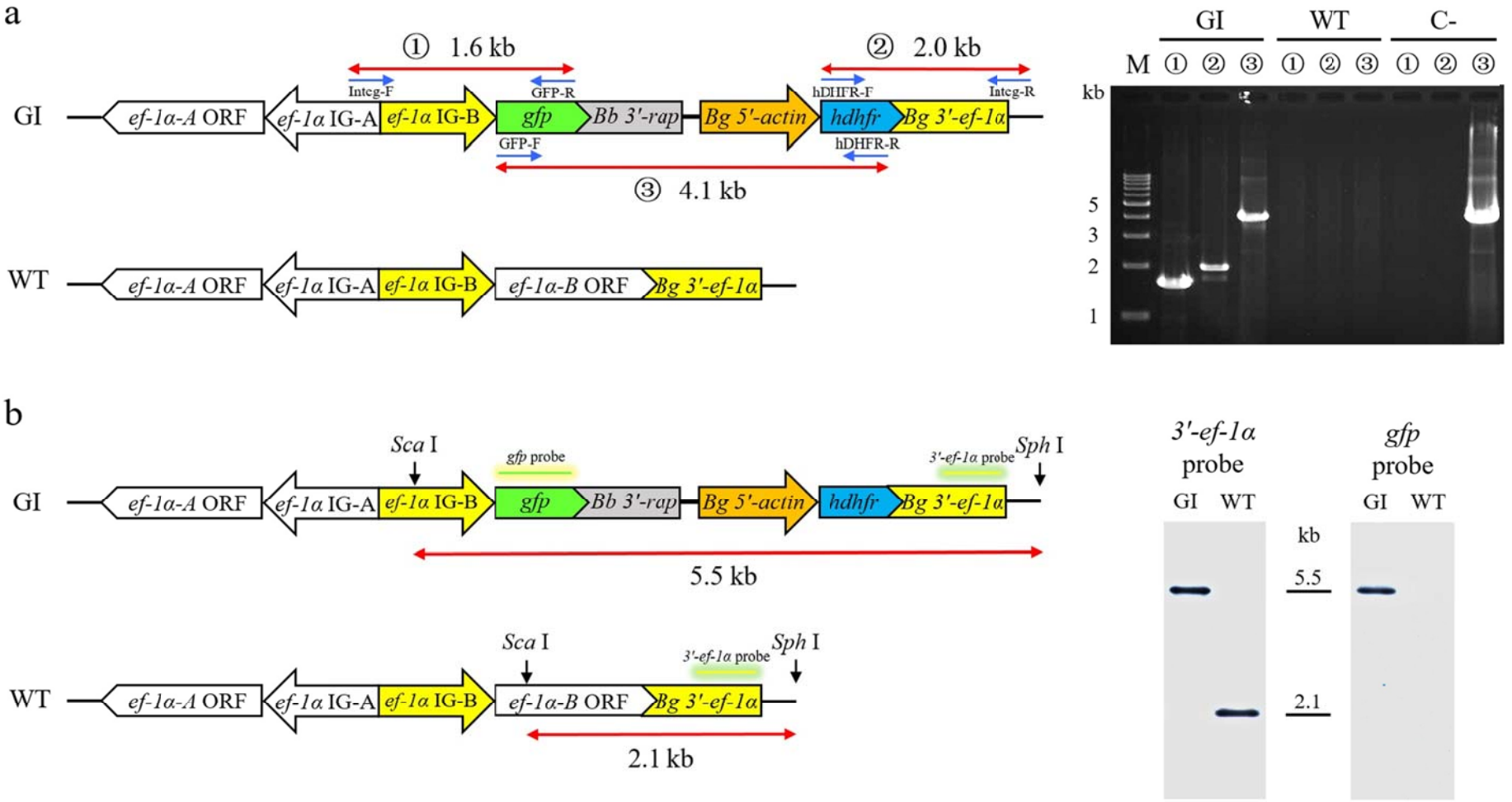
a
① 1.6 kb
② 2.0 kb
Integ-F
GFP-R
hDHFR-F
Integ-R
GI
ef-1α-A ORF
ef-1α IG-A
ef-1α IG-B
gfp
Bb 3'-rap
Bg 5'-actin
hdhfr
Bg 3'-ef-1α
GFP-F
hDHFR-R
③ 4.1 kb
WT
ef-1α-A ORF
ef-1α IG-A
ef-1α IG-B
ef-1α-B ORF
Bg 3'-ef-1α
GI
WT
C-
M ① ② ③ ① ② ③ ① ② ③
kb
5
3
2
1
b
Sca I
gfp probe
3'-ef-1α probe
Sph I
GI
ef-1α-A ORF
ef-1α IG-A
ef-1α IG-B
gfp
Bb 3'-rap
Bg 5'-actin
hdhfr
Bg 3'-ef-1α
5.5 kb
Sca I
3'-ef-1α probe
Sph I
WT
ef-1α-A ORF
ef-1α IG-A
ef-1α IG-B
ef-1α-B ORF
Bg 3'-ef-1α
2.1 kb
3'-ef-1α probe
GI WT
gfp probe
GI WT
kb
5.5
2.1

Fig. 8. Confirmation of integration of pBS-EGRADE into the *ef-1α* locus. a, Schematic diagram and results of PCR to confirm the integration of pBS-EGRADE into the *ef-1α* locus. PCR-1, -2 and -3 were done with primer sets Integ-F/GFP-R, hDHFR-F/Integ-R and GFP-F/hDHFR-R, respectively. b, Schematic diagram and Southern blot analysis to confirm the integration of pBS-EGRADE into *ef-1α* locus. Two µg of samples genomic DNA were digested with *Sca* I and *Sph* I, and hybridized with *3′-ef-1α* and *gfp* probes. *Abbreviations*: GI, genome-integrated; WT, wild type; C-, pBS-EGRADE plasmid control

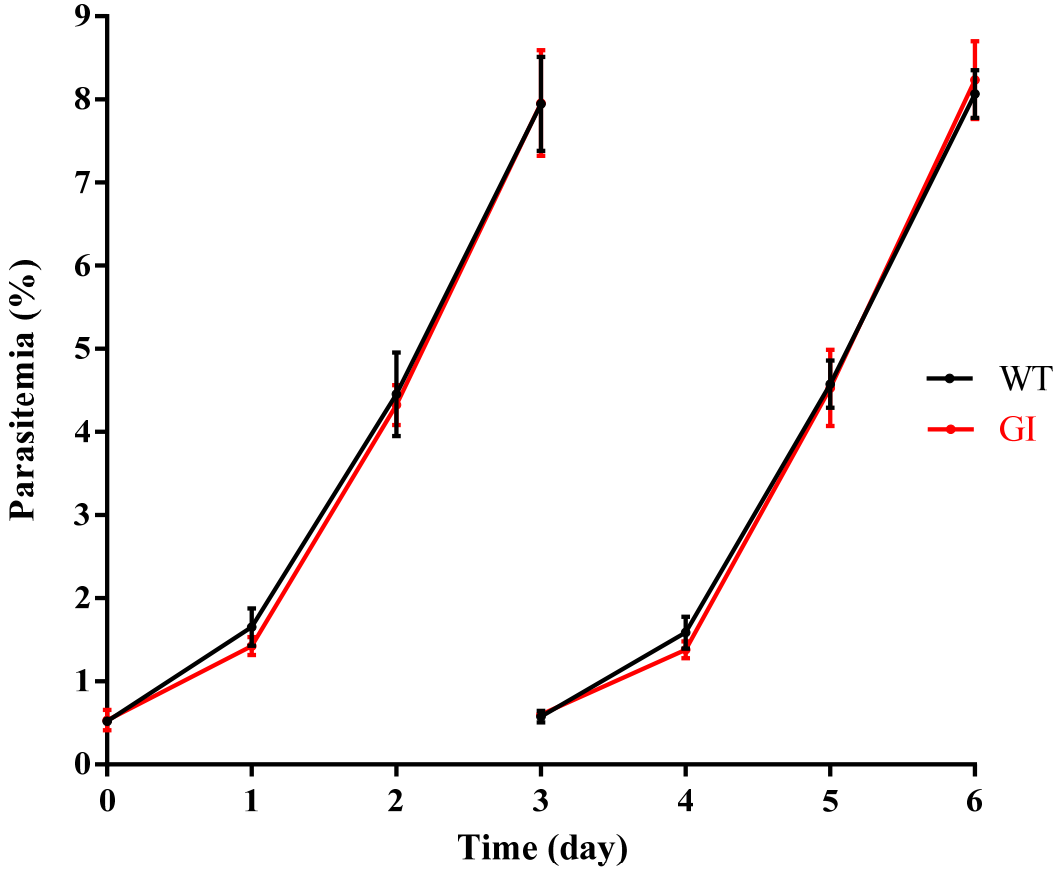

Fig. 9. Growth curves of wild type (WT) and genome integrated (GI) parasites. WT and GI parasites were maintained by sub-culturing every 3 days and parasitemia were monitored daily. All data are expressed as means ± SD of triplicate cultures.

General discussion

Important constraints to research on parasite has included the difficulties associated with the maintenance of *in vitro* culture, promoter, drug selectable marker and transfection system (Suarez and McElwain, 2010). *Babesia gibsoni in vitro* culture was established in 2002 (Sunaga et al., 2002), which is widely used in *B. gibsoni* research and strongly supported the present study.

All previously established transfection systems for *Babesia* focused on bovine *Babesia* species, which were transfected using Gene Pulser Xcell™ Electroporation system (Bio-Rad, USA) and AMAXA Nucleofector™ 2b device (Lonza, Germany) (Suarez and McElwain, 2009; Hakimi et al., 2016; Silva et al., 2016). However, parasite lysates did not show any luciferase activity, indicating failure in introduction of the plasmids into the parasite due to unsuitable buffer and transfection program. This suggests that program FA113 and buffer SF constitutes the best condition among the programs and buffers tested for transfection of *B. gibsoni*. The present method based on 4D Nucleofector™ may provide a more suitable transfection system for non-bovine *Babesia* parasites, such as *B. gibsoni*. The rapid *in vitro* aging of canine erythrocytes (Lehtinen et al., 2008) may play an important role in restricting a successful transfection. Therefore, to avoid the rapid aging of canine erythrocytes, I strongly suggest sub-culturing every week by fresh RBCs after transfection.

Of the 12 promoters identified in this study, *Bg 5′-actin* promoted higher luciferase activity than other 11 promoters, including *Bg 5′-ef-1α, Bb 5′-ef-1α* and *Bb 5′-actin* which were previously reported. Interestingly, *Bg 5′-actin* promoter resulted in a higher luciferase activity in *B. bovis* than in *B. gibsoni*. This may be due either to *Bg 5′-actin* promoter having particular affinity for *B. bovis* transcription factors or to

B. bovis lacking of proper regulatory signals for the regulation of the activity of *Bg 5′-actin* promoter. This result also indicates that heterologous promoter function widely exists between *B. gibsoni* and *B. bovis*. The promoters can recognize the transcription or translation system mutually. These results will provide considerable flexibility in the future construction of plasmid vectors to be used for transfection systems in *Babesia* species.

In this study, I also employed a WR99210/*hdhfr* selection system for *B. gibsoni stable transfection.* The IC_{50} of WR99210 against *B. gibsoni* was 1.1 nM, which is similar to *B. bovis* (1 nM) (Asada et al., 2012) and almost twice that of the one reported for *B. ovata* (0.56 nM) (Hakimi et al., 2016). The transfected parasite selected with WR99210/*hdhfr* emerged as early as two weeks after adding the drug, indicating the suitability of this selection system for *stable transfection of B. gibsoni. B. bovis 3′-rap* was successfully used as terminator in this study. This result is consistent with my previous work (Chapter 2), confirming that *Bb 3′-rap* heterologous terminator is fully functional in *B. gibsoni*. These findings provide considerable flexibility in the construction of plasmid vectors to be used for transfection systems in *Babesia* species.

Overall, a host-*Babesia* infection model may be easier to achieve using canine *Babesia* rather than bovine *Babesia* because using dogs for animal experiments is more feasible than using cattle. The urgently needed genome edited host-*Babesia* infection model may help us monitor transmission *in vivo*, investigate mechanisms of infection and immunity, and also improve the development of novel strategies for controlling babesiosis. Transfection systems improve our understanding of the molecular biology of parasites and pave the way for genetic manipulation (Suarez et

al., 2017). The application of transfection systems can also lead to a better understanding of the mechanisms underlying drug resistance and provide novel information for vaccine development and drug target discovery (Suarez and Noh, 2011). Therefore, due to the lack of techniques for the genetic manipulation of *B. gibsoni*. In order to fill this gap, I describe herein the development of a stable transfection system for *B. gibsoni*.

General summary

The main objectives of the present study is showing as follow: Firstly, to establish a useful transient transfection system of *B. gibsoni*, towards a stable transfection method of *B. gibsoni*. Secondly, to identify and characterize of interchangeable cross-species functional promoters between *B. gibsoni* and *B. bovis*. Thirdly, to establish a stable transfection system for *B. gibsoni*, towards a further genome editing of *B. gibsoni*.

In chapter 1, I established the successful transient transfection of *B. gibsoni*. The plasmid containing the firefly luciferase reporter gene (pBS-ELA) was transfected into *B. gibsoni* by an AMAXA 4D Nucleofector™ device. Transfection using program FA113 and Lonza buffer SF showed the highest luciferase expression. Twenty micrograms of plasmid produced the highest relative transfection efficiency. The fluorescent protein-expressing parasites were determined by GFP-containing plasmid (pBS-EGA) at 48 and 72 hours post transfection. This finding is the first step towards a stable transfection method for *B. gibsoni*, which may contribute to a better understanding of the biology of the parasite.

In chapter 2, I investigated 6 homologous and 6 heterologous promoters for *B. gibsoni* and *B. bovis* and identified novel interchangeable cross-species functional promoters between *B. gibsoni* and *B. bovis.* Ten out of twelve promoters had heterologous promoter function. In particular, *Bg 5′-ef-1α* and *Bg 5′-actin* heterologous promoters resulted in a significantly higher luciferase activity than *Bb 5′-ef-1α* homologous promoter in *B. bovis*. The present study showed that *Bg 5′-actin* promoted the highest luciferase activity in both *B. gibsoni* and *B. bovis.* The study

further indicates that heterologous promoter function widely exists between *B. gibsoni* and *B. bovis*. This finding is an important step for future stable transfection construct design and for the production of vaccines based on transfected *B. gibsoni* and *B. bovis* parasites.

In chapter 3, I generated a plasmid construct in which the 5′-intergenic (IG) region-B of the *ef-1α* gene (5′-*ef-1α*) drives the *gfp* reporter gene, and the 5′-*actin* promotes the expression of the selection marker *hdhfr*. The plasmid was designed for integration into the *ef-1α* locus of *B. gibsoni* genome by double cross-over homologous recombination. Linearized plasmid was transfected by 4D Nucleofector^{TM} into *in vitro* cultured *B. gibsoni* and 10 nM WR99210 was added for drug selection two days after transfection. GFP-expressing parasites were observed by fluorescence microscopy as early as two weeks after drug selection, and consistently expressed GFP for more than 3 months without drug pressure. Genome integration was confirmed by PCR, sequencing and Southern blot analysis. This finding will facilitate functional analysis of *Babesia* genomes using genetic manipulation and will serve as a foundation for the development of tick-*Babesia* and host-*Babesia* infection models.

In summary, I established the transient and stable transfection systems of *B. gibsoni* parasite, which successfully integrated exogenous genes into the *B. gibsoni* genome. The genetic manipulation method describing in this study will serve as the knockout parasite construction, which is widely used in analyzing the gene function and drug target discovery.

和文要約

マダニに媒介される赤血球内寄生原虫であるバベシア属には約 100 種類同定されているが、牛に寄生する３種類（*B. bovis*、*B. bigemina*、*B. ovata*）のみについて遺伝子組換え方法が確立されている。他のバベシアについても遺伝子組換え方法の導入が求められている。そこで、日本を含むアジア地域で犬に広範に流行し、ペット産業に深刻な被害を与えている、イヌバベシア（*B. gibsoni*）について遺伝子組換え方法の確立を試みた。

第 1 章では外来遺伝子一過性発現虫体の作製を試みた。*B. gibsoni* のゲノムデータベースより伸長因子 α（ef-1α）のプロモーター領域を特定し、緑色蛍光タンパク質（GFP）遺伝子またはルシフェラーゼ（Luc）遺伝子発現プラスミドを構築した。最初は、これらのプラスミドをウシバベシアで報告された方法でトランスフェクションを行ったところ、いずれも成功しなかった。そこで、種々のトランスフェクション方法を検討したところ、Lonza 社製の AMAXA 4D Nucleofector™装置用と特定の緩衝液を用いたところ、GFP または Luc 遺伝子一過性発現虫体の作製に成功した。

第 2 章ではプロモーターの探索を行った。上記の伸長因子 α プロモーターを含む 6 種類のプロモーター領域の制御下で Luc 発現プラスミドを作製し、異なるプロモーターの活性を測定したところ、アクチン（actin）プロモーターが最も強い活性を有することが判明した。また、*B. gibsoni* と *B. bovis* の ef-1α と actin プロモーターをそれぞれ、異種虫体に導入してそのプロモーター活性を調べた

ところ、これらのプロモーターは異種虫体においても機能することを突き止めた。

第 3 章では外来遺伝子安定発現虫体の作製を行った。ゲノムの標的部位に外来遺伝子を導入し、安定発現を実現するために、ef-1α 遺伝子の上流と下流ゲノム断片を発現ユニット（ef-1α プロモーター：GFP ORF：ターミネーター：actin プロモーター：dhfr ORF：ef-1α ターミネーター）の両側にもつプラスミド（注 : dhfr、ピリメタミン薬剤耐性遺伝子）を構築し、虫体に導入した後に、薬剤選択を行ったところ、相同組み換えにより GFP 遺伝子と dhfr 遺伝子が標的ゲノム部位に組み込まれた安定発現虫体の作製に成功した。また、標的部位の ef-1α 遺伝子がノックアウト（KO）されたことも確認した（*B. gibsoni* には ef-1α 遺伝子 2 コピー存在し、そのうち 1 コピーのみが KO された）。

以上のように、この研究では、ウシバベシア以外のバベシア種では、初めてイヌバベシア原虫の遺伝子組換え法の確立に成功した。今回確立した方法により、イヌバベシア原虫に自由自在に外来遺伝子を導入でき、また、標的遺伝子を簡単に KO することが出来るようになった。今後、イヌバベシア原虫の病原性遺伝子の特定・KO による、虫体の弱毒化と、この弱毒化虫体をベクターとした新規組換えワクチン開発への応用が期待される。

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References

Abd Rani PA, Irwin PJ, Coleman GT, Gatne M, Traub RJ. 2011. A survey of canine tick-borne diseases in India. Parasit Vectors. 4, 141.

Abdela N, Jilo K. 2016. Bovine babesiosis and its current status in ethiopia: A systemic review. Adv Biol Res. 10, 138–146.

Adamson R, Lyons K, Sharrard M, Kinnaird J, Swan D, Graham S, Shiels B, Hall R. 2001. Transient transfection of *Theileria annulata*. Mol Biochem Parasitol. 114, 53–61.

Asada M, Tanaka M, Goto Y, Yokoyama N, Inoue N, Kawazu SI. 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.

Asada M, Yahata K, Hakimi H, Yokoyama N, Igarashi I, Kaneko O, Suarez CE, Kawazu SI. 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, e0125993.

Azevedo MF, Del Portillo HA. 2007. Promoter regions of *Plasmodium vivax* are poorly or not recognized by *Plasmodium falciparum*. Malar J. 6, 20.

Baldani CD, Machado RZ, Raso TF, Pinto AA. 2007. Serodiagnosis of *Babesia equi* in horses submitted to exercise stress. Pesqui Vet Bras. 27, 179–183.

Baneth G. 2018. Antiprotozoal treatment of canine babesiosis. Vet Parasitol. 254, 58–63.

Barrangou R. 2015. Diversity of CRISPR-Cas immune systems and molecular machines. Genome Biol. 16, 247.

Bhaskaran Ravi L, Bhoopathy D, Subbiah V. 2016. Escalations in the incidence of canine babesiosis over a period of 8 years (2006-2013) in Chennai, Tamil Nadu, India. J Parasit Dis. 40, 1239–1242.

Birkenheuer AJ, Levy MG, Savary KC, Gager RB, Breitschwerdt EB. 1999. *Babesia gibsoni* infections in dogs from North Carolina. J Am Anim Hosp Assoc. 35, 125–128.

Bonnet S, Brisseau N, Hermouet A, Jouglin M, Chauvin A. 2009. Experimental *in vitro* transmission of *Babesia* sp. (EU1) by *Ixodes ricinus*. Vet Res. 40, 21.

Bortesi L, Fischer R. 2015. The CRISPR/Cas9 system for plant genome editing and beyond. Biotechnol Adv. 33, 41–52.

Boustani MR, Gelfand JA. 1996. Babesiosis. Clin Infect Dis. 22, 611–615.

Brayton KA, Lau AOT, Herndon DR, Hannick L, Kappmeyer LS, Berens SJ, Bidwell SL, Brown WC, Crabtree J, Fadrosh D, Feldblum T, Forberger HA, Haas BJ, Howell JM, Khouri H, Koo H, Mann DJ, Norimine J, Paulsen IT, Radune D, Ren Q, Smith RK, Suarez CE, White O, Wortman JR, Knowles DP, McElwain TF, Nene VM. 2007. Genome sequence of *Babesia bovis* and comparative analysis of apicomplexan hemoprotozoa. PLoS Pathog. 3, e148.

Cao J, Yang Q, Zhang J, Zhou Y, Zhang H, Gong H, Zhou J. 2015. Seroprevalence survey of *Babesia gibsoni* infection and tick species in dogs in East China. Vet Parasitol. 214, 12–15.

Casapulla R, Baldi L, Avallone V, Sannino R. 1998. Canine piroplasmosis due to *Babesia gibsoni*: Clinical and morphological aspects the hatching ability of *in vitro* produced buffalo (*Bubalus bubalis*) embryos. Vet Res. 142, 168–169.

Chauvin A, Moreau E, Bonnet S, Plantard O, Malandrin L. 2009. *Babesia* and its hosts: Adaptation to long-lasting interactions as a way to achieve efficient transmission. Vet Res. 40, 37.

Crabb BS, Cowman AF. 1996. Characterization of promoters and stable transfection by homologous and nonhomologous recombination in *Plasmodium falciparum*. Proc Natl Acad Sci U S A. 93, 7289–7294.

De Goeyse I, Jansen F, Madder M, Hayashida K, Berkvens D, Dobbelaere D, Geysen D. 2015. Transfection of live, tick derived sporozoites of the protozoan Apicomplexan parasite *Theileria parva*. Vet Parasitol. 208, 238–241.

de Koning-Ward TF, Speranca MA, Waters AP, Janse CJ. 1999. Analysis of stage specificity of promoters in *Plasmodium berghei* using luciferase as a reporter. Mol Biochem Parasitol. 100, 141–146.

Donald RG, Roos DS. 1993. Stable molecular transformation of *Toxoplasma gondii*: A selectable dihydrofolate reductase-thymidylate synthase marker based on drug-resistance mutations in malaria. Proc Natl Acad Sci U S A. 90, 11703–11707.

Duan Y, Ma G, Huang X, D'Amore PA, Zhang F, Lei H. 2016. The clustered, regularly interspaced, short palindromic repeats-associated endonuclease 9 (CRISPR/Cas9) created MDM2 T309G mutation enhances vitreous-induced

expression of MDM2 and proliferation and survival of cells. J Biol Chem. 291, 16339–16347.

Duh D, Petrovec M, Avsic-Zupanc T. 2001. Diversity of *Babesia* infecting European sheep ticks (*Ixodes ricinus*). J Clin Microbiol. 39, 3395–3397.

Eichenberger RM, Riond B, Willi B, Hofmann-Lehmann R, Deplazes P. 2016. Prognostic markers in acute *Babesia canis* infections. J Vet Intern Med. 30, 174–182.

Fukumoto S, Suzuki H, Igarashi I, Xuan X. 2005. Fatal experimental transplacental *Babesia gibsoni* infections in dogs. Int J Parasitol. 35, 1031–1035.

Fukumoto S, Xuan X, Inoue N, Igarashi I, Sugimoto C, Fujisaki K, Nagasawa H, Mikami T, Suzuki H. 2003. Molecular characterization of a gene edcoding a 29-kDa cytoplasmic protein of *Babesia gibsoni* and evaluation of its diagnostic potentiality. Mol Biochem Parasitol. 131, 129–136.

Fukumoto S, Xuan X, Takabatake N, Igarashi I, Sugimoto C, Fujisaki K, Nagasawa H, Mikami T, Suzuki H. 2004. Inhibitory effect of antiserum to surface antigen P50 of *Babesia gibsoni* on growth of parasites in severe combined immunodeficiency mice given canine red blood cells. Infect Immun. 72, 1795–1798.

Ganesan SM, Morrisey JM, Ke H, Painter HJ, Laroiya K, Phillips MA, Rathod PK, Mather MW, Vaidya AB. 2011. Yeast dihydroorotate dehydrogenase as a new selectable marker for *Plasmodium falciparum* transfection. Mol Biochem Parasitol. 177, 29–34.

Gondard M, Cabezas-Cruz A, Charles RA, Vayssier-Taussat M, Albina E, Moutailler S. 2017. Ticks and tick-Borne pathogens of the caribbean: Current understanding

and future directions for more comprehensive surveillance. Front Cell Infect Microbiol. 7, 490.

Goo YK, Jia H, Aboge GO, Terkawi MA, Kuriki K, Nakamura C, Kumagai A, Zhou J, Lee EG, Nishikawa Y, Igarashi I, Fujisaki K, Xuan X. 2008. *Babesia gibsoni*: Serodiagnosis of infection in dogs by an enzyme-linked immunosorbent assay with recombinant BgTRAP. Exp Parasitol. 118, 555–560.

Goo YK, Xuan X. 2014. New molecules in *Babesia gibsoni* and their application for diagnosis, vaccine development, and drug discovery. Korean J Parasitol. 52, 345–353.

Goonewardene R, Daily J, Kaslow D, Sullivan TJ, Duffyt P, Carter R, Mendist K, Wirth D. 1993. Transfection of the malaria parasite and expression of firefly luciferase. Proc Natl Acad Sci U S A. 90, 5234–5236.

Hakimi H, Yamagishi J, Kegawa Y, Kaneko O, Kawazu SI, Asada M. 2016. Establishment of transient and stable transfection systems for *Babesia ovata*. Parasit Vectors. 9, 171.

Halos L, Lebert I, Abrial D, Danlois F, Garzik K, Rodes D, Schillmeier M, Ducrot C, Guillot J. 2014. Questionnaire-bases survey on the distribution and incidence of canine babesiosis in countries of Western Europe. Parasite. 21, 13.

He L, Miao X, Hu J, Huang Y, He P, He J, Yu L, Malobi N, Shi L, Zhao J. 2017. First molecular detection of *Babesia gibsoni* in dogs from Wuhan, China. Front Microbiol. 8, 1577.

Homer MJ, Aguilar-Delfin I, Telford SR, Krause PJ, Persing DH. 2000. Babesiosis. Clin Microbiol Rev. 13, 451–469.

Howe DK, Mercier C, Messina M, Sibley LD. 1997. Expression of *Toxoplasma gondii* genes in the closely-related apicomplexan parasite *Neospora caninum*. Mol Biochem Parasitol. 86, 29–36.

Howe DK, Sibley LD. 1997. Development of molecular genetics for *Neospora caninum*: A complementary system to *Toxoplasma gondii*. Methods. 13, 123–133.

Ikadai H, Tanaka H, Shibahara N, Matsuu A, Uechi M, Itoh N, Oshiro S, Kudo N, Igarashi I. 2004. Molecular evidence of infections with *Babesia gibsoni* parasites in Japan and evaluation of the diagnostic potential of a loop-mediated isothermal amplification method. J Clin Microbiol. 42, 2465–2469.

Imre M, Farkas R, Ilie MS, Imre K, Dărăbuş G. 2013. Survey of babesiosis in symptomatic dogs from Romania: Occurrence of *Babesia gibsoni* associated with breed. Ticks Tick Borne Dis. 4, 500–502.

Inokuma H, Yoshizaki Y, Matsumoto K, Okuda M, Onishi T, Nakagome K, Kosugi R, Hirakawa M. 2004. Molecular survey of *Babesia* infection in dogs in Okinawa, Japan. Vet Parasitol. 121, 341–346.

Irwin PJ. 2009. Canine babesiosis: From molecular taxonomy to control. Parasit Vectors. 2, S4.

Islam MK, Tsuji N, Miyoshi T, Alim MA, Huang X, Takeshi H, Fujisaki K. 2009. The Kunitz-like modulatory protein haemangin is vital for hard tick blood-feeding success. PLoS Pathog. 5, e1000497.

Iwakami S, Ichikawa Y, Inokuma H. 2014. Molecular survey of *Babesia gibsoni* using *Haemaphysalis longicornis* collected from dogs and cats in Japan. J Vet Med Sci. 76, 1313–1316.

Jacobson LS. 2006. The South African form of severe and complicated canine babesiosis: Clinical advances 1994-2004. Vet Parasitol. 138, 126–139.

Karginov FV, Hannon GJ. 2010. The CRISPR system: small RNA-guided defense in bacteria and archaea. Mol Cell. 37, 7.

Kelleher M, Tomley FM. 1998. Transient expression of β-galactosidase in differentiating sporozoites of *Eimeria tenella*. Mol Biochem Parasitol. 97, 21–31.

Kim K, Soldati D, Boothroyd JC. 1993. Gene replacement in *Toxoplasma gondii* with chloramphenicol acetyltransferase as selectable marker. Science. 262, 911–914.

Konishi K, Sakata Y, Miyazaki N, Jia H, Goo YK, Xuan X, Inokuma H. 2008. Epidemiological survey of *Babesia gibsoni* infection in dogs in Japan by enzyme-linked immunosorbent assay using *B. gibsoni* thrombospondin-related adhesive protein antigen. Vet Parasitol. 155, 204–208.

Kuang D, Qiao J, Li Z, Wang W, Xia H, Jiang L, Dai J, Fang Q, Dai X. 2017. Tagging to endogenous genes of *Plasmodium falciparum* using CRISPR/Cas9. Parasit Vectors. 10, 595.

Kusakisako K, Maeda H, Galay RL, Matsuo T, Tsujio M, Umemiya-Shirafuji R, Mochizuki M, Fujisaki K, Tanaka T. 2015. The vector potential of *Haemaphysalis longicornis* ticks for *Babesia microti* parasites under experimental condition. J Protozool Res 25, 8–17.

Laha R, Bhattacharjee K, Sarmah PC, Das M, Goswami A, Sarma D, Sen A. 2014. *Babesia* infection in naturally exposed pet dogs from a northeastern state (Assam) of india: Detection by microscopy and polymerase chain reaction. J Parasit Dis. 38, 389–393.

Lee MJ, Yu DH, Yoon JS, Li YH, Lee JH, Chae JS, Park J. 2009. Epidemiologic and clinical surveys in dogs infected with *Babesia gibsoni* in South Korea. Vector Borne Zoonotic Dis. 9, 681–686.

Lehtinen LE, Birkenheuer AJ, Droleskey RE, Holman PJ. 2008. *In vitro* cultivation of a newly recognized *Babesia* sp. in dogs in North Carolina. Vet Parasitol. 151, 150–157.

Leisewitz AL, Jacobson LS, De Morais HSA, Reyers F. 2001. The mixed acid-base disturbances of severe canine babesiosis. J Vet Intern Med. 15, 445–452.

Li W, Zhang N, Liang X, Li J, Gong P, Yu X, Ma G, Ryan UM, Zhang X. 2009. Transient transfection of *Cryptosporidium parvum* using green fluorescent protein (GFP) as a marker. Mol Biochem Parasitol. 168, 143–148.

Lin HR, Mei XT, Hong YF, Zhao YB, Guo XN, Yang DJ, Yao DW. 2017. Sequence analysis of the thrombospondin-related adhesive protein gene and heat shock protein 70 gene of *Babesia gibsoni* isolated from dogs in Nanjing, China. Infect Genet Evol. 56, 111–116.

Liu M, Cao S, Zhou M, Wang G, Jirapattharasate C, Adjou Moumouni PF, Iguchi A, Vudriko P, Suzuki H, Soma T, Xuan X. 2016. Genetic variations of four immunodominant antigens of *Babesia gibsoni* isolated from dogs in southwest Japan. Ticks Tick Borne Dis. 7, 298–305.

Maeda H, Hatta T, Alim MA, Tsubokawa D, Mikami F, Matsubayashi M, Miyoshi T, Umemiya-Shirafuji R, Kawazu SI, Igarashi I, Mochizuki M, Tsuji N, Tanaka T. 2016. Establishment of a novel tick-*Babesia* experimental infection model. Sci Rep. 6, 37039.

Malekifard F, Tavassoli M, Yakhchali M, Darvishzadeh R. 2014. Detection of *Theileria equi* and *Babesia caballi* using microscopic and molecular methods in horses in suburb of Urmia, Iran. Vet Res Forum. 5, 129–133.

Mandal M, Banerjee PS, Garg R, Ram H, Kundu K, Kumar S, Kumar GV. 2014. Genetic characterization and phylogenetic relationships based on 18S rRNA and ITS1 region of small form of canine *Babesia* spp. from India. Infect Genet Evol. 27, 325–331.

Mandal M, Banerjee PS, Kumar S, Ram H, Garg R, Pawde AM. 2015. Development of loop-mediated isothermal amplification (LAMP) for detection of *Babesia gibsoni* infection in dogs. Vet Parasitol. 209, 50–55.

Marathe A, Tripathi J, Handa V, Date V. 2005. Human babesiosis--a case report. Indian J Med Microbiol. 23, 267–269.

Meissner M, Brecht S, Bujard H, Soldati D. 2001. Modulation of myosin A expression by a newly established tetracycline repressor-based inducible system in *Toxoplasma gondii*. Nucleic Acids Res. 29, e115.

Miyama T, Inokuma H, Itamoto K, Okuda M, Verdida RA. 2006. Clinical usefulness of antibodies against *Babesia gibsoni* detected by ELISA with recombinant P50. J Vet Med Sci. 68, 1371–1373.

Mokhtar AS, Lim SF, Tay ST. 2013. Molecular detection of *Anaplasma platys* and *Babesia gibsoni* in dogs in Malaysia. Trop Biomed. 30, 345–348.

Mota MM, Thathy V, Nussenzweig RS, Nussenzweig V. 2001. Gene targeting in the rodent malaria parasite *Plasmodium yoelii*. Mol Biochem Parasitol. 113, 271–278.

Pacheco-Lugo L, Díaz-Olmos Y, Sáenz-García J, Probst CM, DaRocha WD. 2017. Effective gene delivery to *Trypanosoma cruzi* epimastigotes through nucleofection. Parasitol Int. 66, 236–239.

Padmanabhan PK, Polidoro RB, Barteneva NS, Gazzinelli RT, Burleigh BA. 2014. Transient transfection and expression of foreign and endogenous genes in the intracellular stages of *Trypanosoma cruzi*. Mol Biochem Parasitol. 198, 100–103.

Paparini A, Senanayake SN, Ryan UM, Irwin PJ. 2014. Molecular confirmation of the first autochthonous case of human babesiosis in Australia using a novel primer set for the beta-tubulin gene. Exp Parasitol. 141, 93–97.

Price AA, Sampson TR, Ratner HK, Grakoui A, Weiss DS. 2015. Cas9-mediated targeting of ciral RNA in eukaryotic cells. Proc Natl Acad Sci U S A. 112, 6164–6169.

Rath D, Amlinger L, Rath A, Lundgren M. 2015. The CRISPR-Cas immune system: biology, mechanisms and applications. Biochimie. 117, 119–128.

Rudolf I, Golovchenko M, Šikutová S, Rudenko N, Grubhoffer L, Hubálek Z. 2005. *Babesia microti* (Piroplasmida: Babesiidae) in nymphal *Ixodes ricinus* (Acari: Ixodidae) in the Czech Republic. Folia Parasitol. 52, 274–276.

Schnittger L, Rodriguez AE, Florin-Christensen M, Morrison DA. 2012. *Babesia*: A world emerging. Infect Genet Evol. 12, 1788–1809.

Scott JD, Scott CM. 2018. Human babesiosis caused by *Babesia duncani* has widespread distribution ascorss Canada. Healthcare. 6, e49.

Shmakov SA, Sitnik V, Makarova KS, Wolf YI, Severnov KV, Koonin EV. 2017. The CRISPR spacer space is dominated by sequences from species-specific mobilomes. MBio. 8, e01397–17.

Sidik SM, Huet D, Ganesan SM, Huynh MH, Wang T, Nasamu AS, Thiru P, Saeij JPJ, Carruthers VB, Niles JC, Lourido S. 2016. A genome-wide CRISPR screen in *Toxoplasma* identifies essential apicomplexan genes. Cell. 166, 1423–1435.e12.

Silva MG, Knowles DP, Mazuz ML, Cooke BM, Suarez CE. 2018. Stable transformation of *Babesia bigemina* and *Babesia bovis* using a single transfection plasmid. Sci Rep. 8, 6096.

Silva MG, Knowles DP, Suarez CE. 2016. Identification of interchangeable cross-species function of *elongation factor-1 alpha* promoters in *Babesia bigemina* and *Babesia bovis*. Parasit Vectors. 9, 576.

Singh AK, Verma AK, Neha, Tiwari R, Karthik K, Dhama K, Singh SV. 2014. Trends and advances in vaccines against protozoan parasites of veterinary importance: A review. J Biol Sci. 14, 95–109.

Skotarczak B. 2008. Babesiosis as a disease of people and dogs. Molecular diagnostics: A review. Vet Med. 53, 229–235.

Solano-Gallego L, Sainz Á, Roura X, Estrada-Peña A, Miró G. 2016. A review of canine babesiosis: The European perspective. Parasit Vectors. 9, 336.

Soldati D, Boothroyd JC. 1993. Transient transfection and expression in the obligate intracellular parasite *Toxoplasma gondii*. Science. 260, 349–352.

Suarez CE, Bishop RP, Alzan HF, Poole WA, Cooke BM. 2017. Advances in the application of genetic manipulation methods to apicomplexan parasites. Int J Parasitol. 47, 701–710.

Suarez CE, McElwain TF. 2010. Transfection systems for *Babesia bovis*: A review of methods for the transient and stable expression of exogenous genes. Vet Parasitol. 167, 205–215.

Suarez CE, McElwain TF. 2009. Stable expression of a GFP-BSD fusion protein in *Babesia bovis* merozoites. Int J Parasitol. 39, 289–297.

Suarez CE, McElwain TF. 2008. Transient transfection of purified *Babesia bovis* merozoites. Exp Parasitol. 118, 498–504.

Suarez CE, Noh S. 2011. Emerging perspectives in the research of bovine babesiosis and anaplasmosis. Vet Parasitol. 180, 109–125.

Suarez CE, Norimine J, Lacy P, McElwain TF. 2006. Characterization and gene expression of *Babesia bovis elongation factor-1 alpha*. Int J Parasitol. 36, 965–973.

Suarez CE, Palmer GH, LeRoith T, Florin-Christensen M, Crabb B, McElwain TF. 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.

Sunaga F, Namikawa K, Kanno Y. 2002. Continuous *in vitro* culture of erythrocytic stages of *Babesia gibsoni* and virulence of the cultivated parasite. J Vet Med Sci. 64, 571–575.

Terao M, Akter S, Yasin MG, Nakao R, Kato H, Alam MZ, Katakura K. 2015. Molecular detection and genetic diversity of *Babesia gibsoni* in dogs in Bangladesh. Infect Genet Evol. 31, 53–60.

van Dijk MR, Waters AP, Janse CJ. 1995. Stable transfection of malaria parasite blood stages. Science. 268, 1358–1362.

Vannier EG, Diuk-Wasser MA, Ben Mamoun C, Krause PJ. 2015. Babesiosis. Infect Dis Clin North Am. 29, 357–370.

Vinayak S, Pawlowic MC, Sateriale A, Brooks CF, Studstill CJ, Bar-Peled Y, Cipriano MJ, Striepen B. 2015. Genetic modification of the diarrhoeal pathogen *Cryptosporidium parvum*. Nature. 523, 477–480.

Vinkenoog R, Sperança MA, Van Breemen O, Ramesar J, Williamson DH, Ross-MacDonald PB, Thomas AW, Janse CJ, Del Portillo HA, Waters AP. 1998. Malaria parasites contain two identical copies of an *elongation factor 1 alpha* gene. Mol Biochem Parasitol. 94, 1–12.

Wozniak EJ, Barr BC, Thomford JW, Yamane I, McDonough SP, Moore PF, Naydan D, Robinson TW, Conrad PA. 1997. Clinical, anatomic, and immunopathologic characterization of *Babesia gibsoni* infection in the domestic dog (Canis familiaris). J Parasitol. 83, 692–699.

Wright AV, Nuñez JK, Doudna JA. 2016. Biology and applications of CRISPR systems: Harnessing nature's toolbox for genome engineering. Cell. 164. 29–44.

Yao DW, Jiang JY, Yu ZZ, Yao DQ, Yang DJ, Zhao YB. 2014. Canine babesiosis in China caused by *Babesia gibsoni*: A molecular approach. Iran J Parasitol. 9, 163–168.

Yu L, Terkawi MA, Cruz-Flores MJ, Claveria FG, Aboge GO, Yamagishi J, Goo YK, Cao S, Masatani T, Nishikawa Y, Xuan X. 2013. Epidemiological surey of *Babesia bovis* and *Babesia bigemina* infections of cattle in Philippines. J Vet Med Sci. 75, 995–998.

Zheng W, Liu M, Adjou Moumouni PF, Liu X, Efstratiou A, Liu Z, Liu Y, Tao H, Guo H, Wang G, Gao Y, Li Z, Ringo AE, Jirapattharasate C, Chen H. Xuan X. 2017. First molecular detection of tick-borne pathogens in dogs from Jiangxi, China. J Vet Med Sci. 79, 248–254.