Recent Advances in Molecular Genetic Tools for Babesia

Hassan Hakimi 1,*, Masahito Asada 2 and Shin-ichiro Kawazu 1

1 Department of Disease Control, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555, Hokkaido, Japan; skawazu@obihiro.ac.jp
2 Department of Global Cooperation, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555, Hokkaido, Japan; masada@obihiro.ac.jp
* Correspondence: hhakimi@obihiro.ac.jp; Tel.: +81-155-49-5642; Fax: +81-155-49-5643
† Present address: Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843-4467, USA.

Abstract: Development of in vitro culture and completion of genome sequencing of several Babesia parasites promoted the efforts to establish transfection systems for these parasites to dissect the gene functions. It has been more than a decade since the establishment of first transfection for Babesia bovis, the causative agent of bovine babesiosis. However, the number of genes that were targeted by genetic tools in Babesia parasites is limited. This is partially due to the low efficiencies of these methods. The recent adaptation of CRISPR/Cas9 for genome editing of Babesia bovis can accelerate the efforts for dissecting this parasite’s genome and extend the knowledge on biological aspects of erythrocytic and tick stages of Babesia. Additionally, glmS ribozyme as a conditional knockdown system is available that could be used for the characterization of essential genes. The development of high throughput genetic tools is needed to dissect the function of multigene families, targeting several genes in a specific pathway, and finally genome-wide identification of essential genes to find novel drug targets. In this review, we summarized the current tools that are available for Babesia and the genes that are being targeted by these tools. This may draw a perspective for the future development of genetic tools and pave the way for the identification of novel drugs or vaccine targets.

Keywords: Babesia; genome; genetic tools

1. Introduction

Babesia are unicellular, apicomplexan tick-borne parasites that have a great economic impact on the livestock industry, pet animal and wildlife health, and a growing concern of human health due to accidental infections by zoonotic Babesia. The parasites were initially discovered at the end 19th century by Babes in cattle with hemoglobinuria [1]. Since then, more than 100 different Babesia spp. were found to infect a wide range of mammals and are considered to be the second most common blood parasites after trypanosomes [2,3]. Mammalian hosts are infected by sporozoites during the tick blood meal and Babesia parasites exclusively invade and multiply within red blood cells (RBCs) (Figure 1). The sexual stage or gamogony happens in the tick midgut, which is followed by kinetes formation and migration to salivary glands, and ultimately sporozoites production which is called transstadial transmission [4]. These sporozoites can infect the next intermediate host following tick molting. A majority of Babesia spp. except B. microti have transovarial transmission in which the parasites spread from the mother tick to the offspring [5]. Babesiosis can have varying degrees of severity based on the parasite species, age, and immunological status of the host and coinfection with other pathogens [3]. The clinical features of the disease include fever, anemia, hemoglobinuria, jaundice, and splenomegaly and can cause severe complications and fatality in some virulent species [3]. The control strategies consist of treatment of patients, tick control using acaricide, live attenuated
vaccine in case of bovine babesiosis, and soluble parasite antigens-based vaccine for canine babesiosis [6,7]. The emerging resistance to drugs and acaricides and lack of effective vaccines are the main obstacles to controlling babesiosis.

![Image of life cycle of Babesia spp.](image)

**Figure 1.** The life cycle of *Babesia* spp. The infection starts when *Babesia* sporozoites are injected into the mammalian host during the blood meal and directly invade and multiply in the RBCs. A subset of the parasite population transforms into gametocytes in the host or upon taken up by the tick where they produce gamete in the tick midgut. Gametes produce diploid zygotes following fertilization. Zygotes invade midgut epithelium and undergo meiotic division which produces kinetes. Kinetes invade and multiply in several organs including salivary glands which results in transstadial transmission (Ts). In most of *Babesia* spp. except *B. microti*, kinetes invade ovaries and eggs which results in parasite transmission into offspring (transovarial transmission, To).

There is an urgent need to develop new drugs and find vaccine candidates against babesiosis. A better understanding of the biology of *Babesia* spp. facilitates identification and characterization of new vaccine and drug targets and assists to understand the molecular basis of current drug resistance. The advanced progress in functional characterization of related apicomplexan parasites, *Plasmodium* spp. and *Toxoplasma gondii*, can shed light on the conserved genes and pathways in *Babesia*; however, there are numerous unique genes that lack homology in related parasites or model organisms and are *Babesia* specific including several multigene families. Identification of conserved biological pathways across *Babesia* spp. can pave the way for finding pan-*Babesia* drug targets [8]. Several *Babesia* parasites are being adapted to in vitro culture [9–15], which facilitate high-throughput compound screens to find novel drugs [16]. However, the molecular targets of the currently available drugs are lagging mainly due to limited genetic tools for these parasites. The genome sequence of *Babesia bovis*, the causative agent of bovine babesiosis, was first to be released and followed by several other *Babesia* spp [17–21]. This advancement motivated researchers to identify gene regulatory regions and further the establishment of genetic modification techniques [22]. Such tools have been used to study parasite biology in the erythrocytic stage and the identification of tick stage-specific proteins [23–
In this review, we summarized the current tools that are available for genetic modification of Babesia spp. and draw the possible road for future advancement in this field.

2. Genome and Genetic Tools for Babesia

Complete genome sequences of B. bovis, B. microti, B. bigemina, B. divergens, Babesia sp. (Xinjiang), B. canis, and B. ovata are available [17–21,29,30]. A chronological timeline of releasing of Babesia genome and developed genetic tools are shown in Figure 2. The transcriptomes of several Babesia in normal or modified culture conditions, virulent and attenuated strains, and tick stage of the parasite are available [20,26,30–34]. These transcriptome data could be used to show stage-specific gene expression, transcription start site, identification of alternative splicing, and better annotation of the genome. Greater than half of the genes in the genome of Babesia parasites have no predicted function which includes the genus-specific genes and several multigene families [8]. Genetic tools are needed to aid in the functional characterization of these genes.

Episomal expression of the transgene could be performed by transfection of circular plasmid DNA while genome integration of the transgene through single or double-cross-over homologous recombination could be achieved using linear plasmids [35,36]. Transient transfection of B. bovis merozoites was reported shortly after the release of this parasite genome [37]. It was followed by two independent reports of stable transfection for this parasite which blasticidin S deaminase (bsd) and human dihydrofolate reductase (hdhfr) was used for the selection of transgenic parasites which confer resistance to blasticidin S and WR99210, respectively [35,38]. Given that the commercially available WR99210 from Sigma-Aldrich is an isomer of the original product from Jacobus pharmaceutical and not functional for the selection of transgenic parasites expressing hDHFR [39], application of hdhfr/WR99210 is limited to the laboratories that have access to the latter product. However, hdhfr also confers resistance to pyrimethamine and this drug could be used instead of WR99210. These advancements in genetic manipulation of Babesia bovis genome inspired other scientists to establish transfection tools for several other Babesia spp. Transient transfections for validation of promoter activity were established for B. bovis, B.
bigemina, B. ovata, B. gibboni, B. ovis, B. microti and Babesia sp. (Xinjiang) [15,37,40–44]. Additionally, stable transfections were reported for several Babesia parasites including B. ovata, B. gibboni, B. bigemina, and B. microti [41,45–47]. As for most of these parasites, the availability of a robust in vitro culture system was a prerequisite to establishing the genetic tools. Regarding B. microti, although stable transfection has been reported in the in vivo condition, transgenic parasites were enriched using fluorescent-activated cell sorting [47]. It is needed to optimize drugs for the selection of transgenic parasites in future studies.

Given the economic importance, availability of genome and several transcriptome data, and availability of several phenotype assays, main progress on Babesia biology in the tick and the mammalian host has been accomplished using B. bovis. However, of ~3800 genes in the genome of B. bovis, only 13 genes have been targeted for epitope tagging, producing point mutation, or gene disruption (Table 1). Several factors hampered the progress on the application of genetic tools for Babesia spp., such as the low efficiency of the transfection system and limited selectable markers. Bio-Rad electroporation device was initially used to transfer plasmid DNA to parasite nucleus [38] and transfection efficiency was improved using Amaxa nucleofector device [35]. Currently, two selectable markers are available for Babesia parasite, bsd and hdhfr [35,38]. Thus, sequential genetic manipulation or complementation studies are possible. Asada et al. (2015) used both selection systems for studying tpx-1 gene knockout and complementation study in B. bovis [24]. Future application of negative selection brings the possibility of recycling the selection markers to perform sequential gene knockout. Of the targeted genes in B. bovis, elongation factor 1-alpha (ef1-α), thioredoxin peroxidase-1 (tpx-1), rad51 and several tick-stage genes such as hap2, 6-Cys E, and 6-Cys A and B were shown to be dispensable in the erythrocytic stage of the parasite [27,28,35,38,48,49]. These gene loci could be used for knock-in or insertion of a fluorescent reporter gene such as gfp for imaging studies. Genetic tools have been used for endogenous or episomal tagging of genes to confirm the localization of their product [25,26]. To dissect segmental gene conversion through homologous recombination in B. bovis, Mack et al., (2019; 2020) disrupted rad51 gene and showed that it is not essential for parasite growth in vitro. However, these parasites lost homologous recombination-dependent gene integration and showed a reduction of in-situ transcriptional switching [9,48]. Recently, a novel multigene family encoding protein with multi-transmembrane domain (mtm) was discovered and overexpressing studies showed that their expression was linked to blasticidin S resistance [26]. Two proteins, SBP2 truncated copy 11 and BbVEAP, were shown to affect cytoadhesion of iRBCs to endothelial cells, thus are involved in B. bovis virulence [26,50]. Upregulation of SBP2 truncated copy 11 reduced binding of iRBCs to endothelial cells, while knockdown of BbVEAP, VESA1-export associated protein, decreased ridge numbers and abrogated cytoadhesion of iRBCs [26,50]. BbVEAP is the first piroplasm-specific protein shown to be essential for parasite development in the RBC [26]. Perforin like protein 1 (Plp1) was shown to be important for parasite egress where knockout parasites had a growth defect with the appearance of RBCs infected with multiple B. bovis [51]. All these studies have been done in the erythrocytic stage of the parasite, and so far, no conditional tools are available for dissecting gene functions in the tick stage. Establishment of genetic tools and characterization of tick stage-specific promoters can accelerate the identification of genes important for the tick stage and assist in finding novel targets for transmission-blocking vaccines.
Table 1. List of *B. bovis* genes targeted for gene disruption, tagging, or overexpression.

| Gene Product                              | Gene ID                     | Targeted Method       | Phenotype                                                                 | Reference |
|-------------------------------------------|-----------------------------|-----------------------|---------------------------------------------------------------------------|-----------|
| Elongation factor 1-alpha (ef1-α)         | BBOV_IV010620               | Knockout              | Not essential for in vitro growth                                         | [23,35,36,38] |
| Thioredoxin peroxidase 1 (Tpx-1)          | BBOV_II004970               | Knockout              | Not essential for in vitro growth, increased sensitivity to nitrosative stress | [24,25]  |
| Hap2                                      | BBOV_III006770              | Knockout              | Not essential for in vitro growth                                         | [28]      |
| 6-Cys E                                   | BBOV_II006640               | Knockout              | Not essential for in vitro growth                                         | [27]      |
| 6-Cys A and B                             | BBOV_II006600, BBOV_II006610 | Double knockout       | Not essential for in vitro growth                                         | [49]      |
| Thioredoxin peroxidase 1 (Tpx-1)          | BBOV_II004970               | Point mutation        | Not essential for in vitro growth, increased sensitivity to nitrosative stress | [25]      |
| Spherical Body Protein 2 (SBP2) truncated copy 11 | BBOV_III006540 | Knockin into ef1-α locus | Reduction in binding of iRBCs to endothelial cells | [50] |
| Spherical Body Protein 3 (SBP3)           | BBOV_II004210               | Epitope tagging       | Protein localization was confirmed with epitope tagging. Not essential for in vitro growth, increased sensitivity to methylmethane sulfonate, loss of HR-dependent integration, and reduction of in situ transcriptional switching | [25] |
| Rad51                                     | BBOV_II003540               | Knockout              | Reverting blasticidin S resistance                                        | [26]      |
| Multi-transmembrane protein (mtm)         | BBOV_III000010, BBOV_III000060 | Episomal overexpression |                                                                 | [26]      |
| VESA1-export associated protein (Bbveap)  | BBOV_III004280              | Knockdown              | Slow growth, abrogation of cytoadhesion                                    | [26]      |
| Perforin like protein 1 (Plp1)            | BBOV_IV001370               | Knockout              | Lower growth rate *in vitro*                                              | [51]      |
3. Genome Editing Using CRISPR/Cas9

Site-specific nucleases include zinc-finger nuclease (ZFN), transcription activator-like effector nucleases (TALEN), and Clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 that selectively produce a double-strand break at a defined genomic site. CRISPR/Cas9 is an acquired immune response in prokaryotes to protect them against invading bacteriophages [52]. This system had successfully been repurposed for genome editing of several organisms, accelerating and revolutionizing their functional genomics. The double-strand break produced by CRISPR/Cas9 should be repaired, which in many organisms happens through error-prone non-homologous end joining (NHEJ), and produces indels, subsequently disrupting the gene function [53]. Babesia spp. lack NHEJ and need template DNA for the repair of double-strand break of DNA [8]. CRISPR/Cas9 system had been adapted to B. bovis and was shown to be efficient for gene editing purposes such as epitope tagging, the introduction of point mutation, and production of gene knockout [25,26]. As shown in Figure 3A, a single plasmid was used to express Cas9, gRNA and the donor template DNA [25]. The ef1-a bidirectional promoter simultaneously drives the expression of Cas9 and hDHFR, whereas U6 splicosomal RNA promoter was used to drive gRNA. gRNA and ~1 kb donor DNA as a template designed based on the target gene could be inserted into AaRI and BamHI sites in the plasmid, respectively. While this single plasmid transfection system was efficient for gene editing, the authors found the integration of the plasmid into the genome which necessitates the application of negative selection to remove the plasmid backbone and recycle hDHFR for sequential gene editing [25]. Integration of CRISPR plasmid into genome tends to happen when a single plasmid is being used in the rodent malaria parasite, Plasmodium yoelii [54]. Expression of two gRNAs using a novel ribosome-mediated CRISPR system or genome integration of Cas9 together with using linear donor DNA prevents integration of plasmid and allows recycling of drug selection cassette [55,56]. Application of inducible Cas9 and stage-specific expressed Cas9 can enhance our ability to dissect Babesia genome during the erythrocytic or tick stage. Null Cas9 could be employed to precisely guide the epigenetic regulators to the transcription start site to control transcription of the target gene [57]. Recently, a new class of CRISPR/Cas system, Cas13, was identified which targets RNA. Cas13 has been validated for transcriptome engineering such as RNA editing, RNA knockdown, and manipulating RNA splicing [58]. It was shown that Cas13 has favorable efficiencies in mammalian and plant cells with no off-target, unintended knockdown of genes, unlike the RNA interference (RNAi) system making it a promising high-throughput genetic tool for Babesia.
Figure 3. Schematic of CRISPR/Cas9 plasmid and conditional systems to regulate gene expression for Babesia bovis. (A) Cas9 and hdhfr are driven by efl-α bidirectional promoter or intergenic region (IG) while U6 spliceosomal RNA promoter or 5’ noncoding region (5’-NR) drives gRNA expression. The gRNA and donor DNA are inserted into AaRI and BamHI sites in the plasmid, respectively. (B) Indirect immunofluorescence microscopy test of BbVEAP-myc-glmS parasite in the presence (+) or absence (−) of glucosamine, GlcN (α-myc, red and α-SBP4 (control), green). The parasite nuclei were stained with Hoechst 33,342 (Hoechst, blue). Scale bar = 5 μm. (C) Live fluorescence microscopy images of green fluorescent protein-destabilizing domain (GFP-DD)-expressing parasites in the presence (+) or absence (−) of Shield. The parasite nuclei were stained with Hoechst 33,342 (Hoechst, blue). Scale bar = 10 μm. DIC, differential interference contrast.

4. Conditional Knockdown Systems

Given that Babesia genome is haploid in erythrocytic and most of the developmental stages in the tick, conventional knockout systems are not suitable to be used for functional characterization of essential genes. Therefore, conditional systems are needed to dissect the functions of indispensable genes to gain insights into druggable targets. Conditional or inducible expression systems can regulate target expression at the genome, transcriptome, or protein level. There is a single report describing the conditional knockdown of mRNA in B. bovis using self-cleaving ribozyme [26]. The glmS ribozyme from Gram-positive bacteria [59] could be activated by glucosamine-6-phosphate. The knockdown of BbVEAP in the presence of inducer was ~90% at protein level (Figure 3B) [26]. This reduction confirmed the role of BbVEAP in parasite development in the RBC, VESA1 export
and cytoadhesion of iRBCs to endothelial cells [26,60]. Riboswitch system could be simply employed by insertion of glms sequence at 3’ non-coding region of the gene of interest open reading frame, downstream of the stop codon and is a promising method to be used for mRNA knockdown of Babesia. RNAi has been used in one study to evaluate the effects of several genes in B. bovis growth in the culture [61]. Because Babesia parasites lack RNAi machinery [8], the applicability of this system requires further validation.

To study the protein function, protein level could be manipulated by inducing premature degradation by fusing protein to destabilizing domain or translocation of the target protein by a method called knock sideways, KS [62,63]. The advantage of targeting the protein of interest is the fast action of this system that could be leveraged for studying the rapid biological process [64]. We have validated FK506-binding protein (FKBP)-based destabilizing domain (DD) by fusing GFP with DD (Figure 3C, unpublished data). FKBP-DD could be fused to the N- or C-terminus of the target protein. DD could be stabilized by the addition of Shield 1 and in the absence of Shield 1, the target protein degradation will be promoted via the proteasome. The applicability of the DD system for Babesia requires further investigation; however, this system is not suitable for the membrane or secreted proteins that are not accessible to the proteasome in the parasite cytoplasm [64,65].

KS which initially called anchor-away is based on conditional tethering of the protein of interest by rapamycin-dependent dimerization where target protein is fused with FKBP and additionally, FRB is fused to a protein with different cellular localization called mislocalizer [63,66]. The addition of rapamycin results in the relocation of the target protein preventing its function. KS requires prior information regarding localization of target protein, but was shown to be an efficient method to study protein function in several organisms including Plasmodium [66,67]. Thus, it could be the method of choice for studying protein function in Babesia.

Conditional knockout methods for genes are not developed for Babesia. Conditional deletion of a target gene using dimerizable Cre recombinase (DiCre) has been established for Plasmodium spp. and was shown to be efficient for several targets in the in vitro and in vivo models [64,65,68]. This system has two compartments—DiCre, in which its inactive two proteins are fused upon rapamycin addition, and a short targeting sequence called loxP. The loxP sequences are inserted upstream and downstream of the locus, which is targeted by DiCre and excises the locus. Additionally, a split Cas9 that becomes functional after dimerization could be used for conditional induction of double-strand break for genome editing [69].

5. In vitro Culture of Babesia and Transfection

In recent years, there have been major technical advances to genetically manipulate Babesia. The main prerequisite for the establishment of transfection systems for the majority of these parasites is the availability of in vitro culture. Although several Babesia species could be cultured in vitro, these methods are not all well optimized. The primary concern for continuous culture of these parasites is the need for animal serum, cryopreservation methods, and continuous supplement of fresh host erythrocytes. To overcome these challenges, several groups established the application of serum-free mediums, GIT, for B. bovis [70], B. bigemina and B. divergens [71], or replaced animal serum with high-density lipoprotein [72], or Albumax I [73] for B. divergens, or lipid mixture for B. bigemina [74]. While it is known that different batches of host serum have content variations and can modify drug effects on Babesia [70], how these methods affecting transfection efficiencies are unclear. However, standardization of in vitro culture and cryopreservation methods using commercially available products [75] can assure reproducible transfection efficiencies and recoveries of cryopreserved stocks across different laboratories. Current B. microti transfection is based on in vivo experiments and it is hard to maintain this parasite in vitro [76]. Short term in vitro selection was shown to be efficient for generating transgenic rodent malaria parasite, P. berghei [77]. Optimization of in vitro culture for this parasite can open
up the possibility of application of currently used drugs in the in vitro culture system, WR99210 and blasticidin S, for selection of transgenic *B. microti*.

6. Future Perspective

The slow progress in functional characterization of *Babesia* genes is partially due to the low efficiencies of transfection for these parasites, which needs further improvement. Currently, iRBCs are being used for transfection. Large-scale preparation of parasite meronts is available and may improve the efficiency of transfection [60].

Limited conditional tools are available to study essential genes in *Babesia*. Conditional tools that can modulate gene expression at the genome, transcriptome, or protein levels are needed. Additionally, non-homologous end joining (NHEJ) does not exist in piroplasms; thus, high-throughput screening methods such as CRISPR/Cas9 mediated gene disruption [78] are not applicable for these parasites. The development of high-throughput tools such as CRISPR/Cas13 for *Babesia* will pave the way for functional characterization of multigene families and genome-wide functional characterization to identify the essential genes and pathways to prioritize research for drug discovery.

**Author Contributions:** Writing-original draft preparation, H.H. Writing-review and editing, H.H., M.A., and S-I.K. All authors read, edited, and approved the final version of the manuscript.

**Funding:** This study was supported partly by grants from Japan Society for the Promotion of Science to H.H. (15K18783, 19K15983), M.A. (19K06384), S-I.K. (19H03120) and Japan-Czech Republic Research Cooperative Program between JSPS and CAS to S.K. (JPJBP120212501)

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** We are grateful to Shino Yamasaki-Sugi for her assistance with the illustration of *Babesia* lifecycle. This work was conducted at the Joint Usage/Research Center on protozoan diseases, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan.

**Conflicts of Interest:** The authors declare no conflict of interests.

**References**

1. Babes, V. Sur l’he’moglobinurie bacté’rienne du boeuf. *C. R. Acad. Sci.*, **1888**, *107*, 692–694.
2. Homer, M.J.; Aguilar-Delfin, I.; Telford III, S.R.; Krause, P.J.; Persing, D.H. Babesiosis. *Clin Microbiol Rev.* **2000**, *13*(3), 451–69.
3. Schnittger, L.; Rodriguez, A.E.; Florin-Christensen, M.; Morrison, D.A. Babesia: A world emerging. *Infect. Genet. Evol.* **2012**, *12*, 1788–1809, https://doi.org/10.1016/j.meegid.2012.07.004.
4. Jalovecka, M.; Hajdusek, O.; Sojka, D.; Kopacek, P.; Malandrin, L. The Complexity of Piroplasms Life Cycles. *Front. Cell. Infect. Microbiol.* **2018**, *8*, 248, https://doi.org/10.3389/fcimb.2018.00248.
5. Jalovecka, M.; Sojka, D.; Ascencio, M.; Schnittger, L. *Babesia* Life Cycle – When Phylogeny Meets Biology. *Trends Parasitol.* **2019**, *35*, 356–368, https://doi.org/10.1016/j.pt.2019.01.007.
6. Rathinasamy, V.; Poole, W.A.; Bastos, R.G.; Suarez, C.E.; Cooke, B.M. Babesiosis Vaccines: Lessons Learned, Challenges Ahead, and Future Glimpses. *Trends Parasitol.* **2019**, *35*, 622–635, https://doi.org/10.1016/j.pt.2019.06.002.
7. Solano-Gallego, L.; Sainz, Ángel; Roura, X.; Peña, A.E.; Miro, G. A review of canine babesiosis: the European perspective. *Parasites Vectors* **2016**, *9*, 1–18, https://doi.org/10.1186/s13071-016-1596-0.
8. Keroack, C.; Elsworth, B.; Duraisingh, M.T. To kill a piroplasm: genetic technologies to advance drug discovery and target identification in Babesia. *Int. J. Parasitol.* **2019**, *49*, 153–163, https://doi.org/10.1016/j.ijpara.2019.08.005.
9. Mack, E.A.; Tagliamonte, M.S.; Xiao, Y-P.; Quesada, S.; Allred, D.R. *Babesia bovis* Rad51 ortholog influences switching of ves genes but is not essential for segmental gene con-version in antigenic variation. *PLoS Pathog.* **2020**, *16*(8), e1008772.
10. Guan, G.; Ma, M.; Liu, A.; Du, P.; Ren, Q.; Li, Y.; Wang, J.; Liu, Z.; Yin, H.; Luo, J. Continuous in vitro cultivation of a recently identified *Babesia* that infects small ruminants in China. *Vet Parasitol* **2012**, *187*, 371–378, https://doi.org/10.1016/j.vetpar.2012.02.006.
11. Igarashi, I., Avarzed, A., Tanaka, T., Inoue, N., Ito, M., Omata, Y., Saito, A., Suzuki, N., Continuous in vitro cultivation of *Babesia ovata*. *J Protozool Res* **1994**, *4*, 111–118.
12. Väyrynen, R.; Tuomi, J. Continuous in Vitro Cultivation of Babesia Diversgen. Acta Veter- Scand. 1982, 23, 471–472, https://doi.org/10.1116/bf03546800.

13. Vega, C.; Buening, G.; Rodriguez, S.; Carson, C. Cloning of in vitro propagated Babesia bigemina. Vetar- Parasitol. 1986, 22, 223–233, https://doi.org/10.1016/0304-4017(86)90109-3.

14. Levy, M.; Ristic, M. Babesia bovis: continuous cultivation in a microaerophilous stationary phase culture. Science 1980, 207, 1218–1220, https://doi.org/10.1126/science.7355284.

15. Wang, X.W.; J. Liu, J. Yang, J. Zhao-yong, L.V. Liu, A. Li, Y. Li, Y. Lan, H. Liu, G. Luo, J. Guan, G. Yin, H. Establishment of a transient transfection system for Babesia sp. Xinjiang using homologous promoters. Parasitol Res. 2021, 120, 3625–3630, https://doi.org/10.1007/s00436-021-07250-4

16. Rizk, M.A.; El-Sayed, S.A.E.-S.; Nassif, M.; Mosqueda, J.; Xuan, X.; Igarashi, I. Assay methods for in vitro and in vivo anti-Babesia drug efficacy testing: Current progress, outlook, and challenges. Vetar- Parasitol. 2019, 279, 109013, https://doi.org/10.1016/j.vetpar.2019.109013.

17. A. Brayton, K.; Lau, A.; Herndon, D.R.; Hanning, L.; Kappmeyer, L.; Berens, S.J.; Bidwell, S.L.; Brown, W.C.; Crabtree, J.; Fadrosk, D.; et al. Genome Sequence of Babesia bovis and Comparative Analysis of Apicomplexan Hemoproteina. PLOS Pathog. 2007, 3, e1371, https://doi.org/10.1371/journal.ppat.0030148.

18. Cornillot, E.; Hadj-Kaddour, K.; Dassoul, A.; Noel, B.; Ranwez, V.; Vacherie, B.; Augagneur, Y.; Bres, V.; Duclos, A.; Randazzo, S.; et al. Sequencing of the smallest Apicomplexan genome from the human pathogen Babesia microti. Nucleic Acids Res. 2012, 40, 9102–9114, https://doi.org/10.1093/nar/gks700.

19. Jackson, A.P.; Otto, T.; Darby, A.; Ramaprasad, A.; Xia, D.; Echaide, I.E.; Farber, M.; Gahlot, S.; Gamble, J.; Gupta, D.; et al. The evolutionary dynamics of variant antigen genes in Babesia reveal a history of genomic innovation underlying host-parasite interaction. Nucleic Acids Res. 2014, 42, 7113–7131, https://doi.org/10.1093/nar/gku322.

20. Yamagishi, J.; Asada, M.; Hakimi, H.; Tanaka, T.Q.; Sugimoto, C.; Kawazu, S.-I. Whole-genome assembly of Babesia ovata and comparative genomics between closely related pathogens. BMC Genom. 2017, 18, 1–9, https://doi.org/10.1186/s12864-017-4230-4.

21. Guan, G.; Korbonen, P.K.; Young, N.D.; Koehler, A.V.; Wang, T.; Li, Y.; Liu, Z.; Luo, J.; Yin, H.; Gasser, R.B. Genomic resources for a unique, low-virulence Babesia taxon from China. Parasites Vectors 2016, 9, 1–8, https://doi.org/10.1186/s13071-016-1846-1.

22. Suarez, C.E. and T.F. McElwain, Transfection systems for Babesia bovis: a review of methods for the transient and stable expression of exogenous genes. Vet Parasitol. 2010, 170, 205–15.

23. Asada, M.; Goto, Y.; Yahata, K.; Yokoyama, N.; Kawai, S.; Inoue, N.; Kaneko, O.; Kawazu, S.-I. Gliding Motility of Babesia bovis Merozoites Visualized by Time-Lapse Video Microscopy. PLOS ONE 2012, 7, e35227, https://doi.org/10.1371/journ- al.pone.0035227.

24. Asada, M.; Yahata, K.; Hakimi, H.; Yokoyama, N.; Igarashi, I.; Kaneko, O.; Suarez, C.E.; Kawazu, S.-I. Transfection of Babesia bovis by Double Selection with WR9210 and Blastocidin-S and Its Application for Functional Analysis of Thiorodoxin Peroxi- diase-I. PLOS ONE 2015, 10, e0125993, https://doi.org/10.1371/journal.pone.0125993.

25. Hakimi, H.; Ishizaki, T.; Kegawa, Y.; Kaneko, O.; Kawazu, S.-I.; Asada, M. Genome Editing of Babesia bovis Using the CRISPR/Cas9 System. mSphere 2019, 4, e00109-19, https://doi.org/10.1128/mSphere.00109-19.

26. Hakimi, H.; Templeton, T.J.; Sakaguchi, M.; Yamagishi, J.; Miyazaki, S.; Yahata, K.; Uchihashi, T.; Kawazu, S.-I.; Kaneko, O.; Asada, M. Novel Babesia bovis exported proteins that modify properties of infected red blood cells. PLOS Pathog. 2020, 16, e1008917, https://doi.org/10.1371/journal.ppat.1008917.

27. Alzan, H.F.; Silva, M.G.; Davis, W.C.; Herndon, D.R.; Schneider, D.A.; Suarez, C.E. Geno- and phenotypic characteristics of a transfected Babesia bovis 6-Cys-E knockout clonal line. Parasites Vectors 2017, 10, 214, https://doi.org/10.1186/s13071-017-2143-3.

28. Hussein, H.E.; Bastos, R.G.; Schneider, D.A.; Johnson, W.C.; Adham, F.K.; Davis, W.C.; Laughery, J.M.; Herndon, D.R.; Alzan, H.F.; Ueti, M.W.; et al. The Babesia bovis hap2 gene is not required for blood stage replication, but expressed upon in vitro sexual stage induction. PLOS Neglected Trop. Dis. 2017, 11, e0005965–e0005965, https://doi.org/10.1371/journal.pntd.0005965.

29. Cuesta, I.; Gonzalez, L.M.; Estrada, K.; Grande, R.; Zaballos, Angel; Lobo, C.A.; Barrera, J.; Sanchez-Flores, A.; Montero, E.; Takatani, N.; et al. High-Quality Draft Genome Sequence of Babesia divergens, the Etiological Agent of Cattle and Human Babesi- osis. Genome Announc. 2014, 2, e01168-14, https://doi.org/10.1128/genomea.01194-14.

30. Eichenberger, R.M.; Ramakrishnan, C.; Russo, G.; Deplazes, P.; Hehl, A.B. Genome-wide analysis of gene expression and protein secretion of Babesia canis during in-vitro infection identifies potential pathogenicity factors. Sci Rep. 2017, 7(1), 3357.

31. Lau, A.O.; Kalyanaraman, A.; Echaide, I.; Palmer, G.H.; Bock, R.; Pedroni, M.J.; Ramesh Kumar, M.; Ferreira, M.B.; Fletcher, T.I.; McElwain, T.F. Attenuation of virulence in an apicomplexan hemoparasite results in reduced genome diversity at the popu- lation level. BMC Genomics, 2011, 12, 410.

32. Pedroni, M.J.; Sondgeroth, K.S.; Gallego-Lopez, G.M.; Echaide, I.; Lau, A.O. Comparative transcriptome analysis of geographically distinct virulent and attenuated Babesia strains reveals similar gene expression changes through attenuation. BMC Genom. 2013, 14, 763–763, https://doi.org/10.1186/1471-2164-14-763.

33. González, L.M.; Estrada, K.; Grande, R.; Jiménez-Jacinto, V.; Vega-Alvarado, L.; Sevilla, E.; de la Barrera, J.; Cuesta, I.; Zaballos, A.; Bautista, J.M.; et al. Comparative and functional genomics of the protozoan parasite Babesia divergens highlighting the invasion and egress processes. PLoS Negl. Trop. Dis, 2019, 13(8), e0007680.
34. Ueti, M.W.; Johnson, W.C.; Kappmeyer, L.S.; Herndon, D.R.; Mousel, M.R.; Reif, K.E.; Taus, N.S.; Iferon, O.O.; Silva, J.C.; Suarez, C.E.; et al. Comparative analysis of gene expression between Babesia bovis blood stages and kinetes allowed by improved genome annotation. Int. J. Parasitol. 2020, 51, 123–136, https://doi.org/10.1016/j.ijpara.2020.08.006.

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

36. Suarez, C.E.; Johnson, W.C.; Herndon, D.R.; Laughery, J.; Davis, W.C. Integration of a transfected gene into the genome of Babesia bovis occurs by legitimate homologous recombination mechanisms. Mol Biochem Parasitol. 2015, 202(2), 23–28.

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

38. Suarez, C.E.; McElwain, T.F. Stable expression of a GFP-BSD fusion protein in Babesia bovis merozoites. Int. J. Parasitol. 2009, 39, 289–297, https://doi.org/10.1016/j.ijpara.2008.08.006.

39. Remcho, T.P.; Guggilapu, S.D.; Cruz, P.; Nardone, G.A.; Heffernan, G.; O’Connor, R.D.; Bewley, C.A.; Wellem, T.E.; Lane, K.D. Regiosomiserization of Antimalarial Drug WR99210 Explains the Inactivity of a Commercial Stock. Antimicrob. Agents Chemother. 2020, 65, https://doi.org/10.1128/aac.01385-20.

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

41. Hakimi, H.; Yamagishi, J.; Kegawa, Y.; Kaneko, O.; Kawazu, S.-I.; Asada, M. Establishment of transient and stable transfection systems for Babesia ovata. Parasites Vectors 2017, 9, 1–9, https://doi.org/10.1186/s13071-016-1439-z.

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

43. Rosa, C.; Asada, M.; Hakimi, H.; Domingos, A.; Pimentel, M.; Antunes, S. Transient transfection of Babesia ovis using heterologous promoters. Ticks Tick-borne Dis. 2019, 10, 101279, https://doi.org/10.1016/j.ttbdis.2019.101279.

44. Liu, M.; Ji, S.; Rizk, M.A.; Moumouni, P.F.A.; Galon, E.M.; Li, J.; Li, Y.; Zheng, W.; Benedicto, B.; Tumwebaze, M.A.; et al. Transient Transfection of the Zoontic Parasite Babesia microti. Pathogens 2020, 9, 108, https://doi.org/10.3390/pathogens9020108.

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

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

47. Jaiyjan, D.K.; Govindasamy, K.; Singh, J.; Bhattacharya, S.; Singh, A.P. Establishment of a stable transfection method in Babesia microti and identification of a novel bidirectional promoter of Babesia microti. Sci. Rep. 2020, 10, 1–12, https://doi.org/10.1038/s41598-020-72499-3.

48. Mack, E.A.; Xiao, Y.-P.; Allred, D.R. Knockout of Babesia bovis rad51 ortholog and its complementation by expression from the BbACC5 artificial chromosome platform. PLOS ONE 2019, 14, e0215882, https://doi.org/10.1371/journal.pone.0215882.

49. Alzan, H.; Cooke, B.M.; Suarez, C.E. Transgenic Babesia bovis lacking 6-Cys sexual-stage genes as the foundation for non-transmissible live vaccines against bovine babesiosis. Ticks Tick-borne Dis. 2019, 10, 722–728, https://doi.org/10.1016/j.ttbdis.2019.01.006.

50. Lopez, G.G.; Lau, A.O.; O’Connor, R.M.; Ueti, M.W.; Cooke, B.M.; Laughery, J.M.; Graça, T.; Madsen-Bouterse, S.; Oldiges, D.P.; Allred, D.; et al. Up-regulated expression of spherical body protein 2 truncated copy 11 in Babesia bovis is associated with reduced cytoadhesion to vascular endothelial cells. Int. J. Parasitol. 2018, 48, 127–137, https://doi.org/10.1016/j.ijpara.2018.05.015.

51. Paolletta, M.S.; Laughery, J.M.; Arias, L.S.L.; Ortiz, J.M.J.; Montenegro, V.N.; Petrigh, R.; Ueti, M.W.; Suarez, C.E.; Farber, M.D.; Wilkowsky, S.E. The key to egress? Babesia bovis perforin-like protein 1 (PLP1) with hemolytic capacity is required for blood stage replication and is involved in the exit of the parasite from the host cell. Int. J. Parasitol. 2021, 51, 643–658, https://doi.org/10.1016/j.ijpara.2020.12.010.

52. Jin, M.; Chylnski, K.; Fonfara, I.; Hauer, M.; Doudna, J.A.; Charpentier, E. A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. Science 2012, 337, 816–821, https://doi.org/10.1126/science.1225829.

53. Lee, A.H.; Symington, L.S.; Fidock, D.A. DNA Repair Mechanisms and Their Biological Roles in the Malaria Parasite Plasmodium falciparum. Microbiol. Mol. Biol. Rev. 2014, 78, 469–486, https://doi.org/10.1128/mmbrr.00059-13.

54. Zhang, C.; Gao, H.; Yang, Z.; Jiang, Y.; Li, Z.; Wang, X.; Xiao, B.; Su, X.; Cui, H.; Yuan, J. CRISPR/Cas9 mediated sequential editing of genes critical for ookinete motility in Plasmodium yoelii. Mol. Biochem. Parasitol. 2016, 212, 1–8, https://doi.org/10.1016/j.molbiopara.2016.12.010.

55. Walker, M.P.; Lindner, S.E. Ribozyme-mediated, multiplex CRISPR gene editing and CRISPR interference (CRISPRi) in rodent-infesting Plasmodium yoelii. J. Biol. Chem. 2019, 294, 9555–9566, https://doi.org/10.1074/jbc.ca118.070121.

56. Shinzawa, N.; Nishi, T.; Hiyoshi, F.; Motoooka, D.; Yuda, M.; Iwanaga, S. Improvement of CRISPR/Cas9 system by transfecting Cas9-expressing Plasmodium berghei with linear donor template. Commun. Biol. 2020, 3, 1–13, https://doi.org/10.1038/s42003-020-01138-2.

57. Xiao, B.; Yin, S.; Hu, Y.; Sun, M.; Wei, J.; Huang, Z.; Wen, Y.; Dai, X.; Chen, H.; Mu, J.; et al. Epigenetic editing by CRISPR/dCas9 in Plasmodium falciparum. Proc. Natl. Acad. Sci. 2018, 116, 255–260, https://doi.org/10.1073/pnas.1813542116.
58. Konermann, S.; Lotfy, P.; Brideau, N.J.; Oki, J.; Shokhirev, M.N.; Hsu, P.D. Transcriptome Engineering with RNA-Targeting Type VI-D CRISPR Effectors. Cell 2018, 173, 665–676.e14, https://doi.org/10.1016/j.cell.2018.02.033.

59. Winkler, W.C.; Nahvi, A.; Roth, A.; Collins, J.A.; Breaker, R. Control of gene expression by a natural metabolite-responsive ribozyme. Nature 2004, 428, 281–286, https://doi.org/10.1038/nature02362.

60. Hakimi, H.A., M. Ishizaki, T. Kawazu, S., Isolation of viable Babesia bovis merozoites to study parasite invasion. Scientific reports, 2021, 11, 16959.

61. AbouLaila, M.Y., N. Igashiri, I, RNA INTERFERENCE (RNAI) FOR SOME GENES FROM BABESIA BOVIS. Research Journal of Applied Biotechnology 2016, 2, 81–92.

62. Banaszynski, L.A.; Chen, L.-C.; Maynard-Smith, L.A.; Ooi, A.G.L.; Wandless, T.J. A Rapid, Reversible, and Tunable Method to Regulate Protein Function in Living Cells Using Synthetic Small Molecules. Cell 2006, 126, 995–1004, https://doi.org/10.1016/j.cell.2006.07.025.

63. Haruki, H.; Nishikawa, J.; Laemmli, U.K. The Anchor-Away Technique: Rapid, Conditional Establishment of Yeast Mutant Phenotypes. Mol. Cell 2008, 31, 925–932, https://doi.org/10.1016/j.molcel.2008.07.020.

64. Kudyba, H.M.; Cobb, D.W.; Vega-Rodriguez, J.; Muralidharan, V. Some conditions apply: Systems for studying Plasmodium falciparum protein function. PLOS Pathog. 2021, 17, e1009442, https://doi.org/10.1371/journal.ppat.1009442.

65. de Koning-Ward, T.; Gilson, P.R.; Crab, B.S. Advances in molecular genetic systems in malaria. Nat. Rev. Genet. 2015, 13, 373–387, https://doi.org/10.1038/nrmicro3450.

66. Birnbaum, J.; Flemming, S.; Reichard, N.; Soares, A.B.; Mesén-Ramírez, P.; Jonscher, E.; Bergmann, B.; Spielmann, T. A genetic system to study Plasmodium falciparum protein function. Nat. Methods 2017, 14, 450–456, https://doi.org/10.1038/nmeth.4223.

67. Hughes, K.R. and A.P. Waters, Rapid inducible protein displacement in Plasmodium vivax and in vitro using knocksideways technology. Wellcome Open Res, 2017, 2, 18.

68. Knuepfer, E.; Napierkowska, M.; van Ooij, C.; Holder, A.A. Generating conditional gene knockouts in Plasmodium - a toolkit to produce stable DiCre recombinase-expressing parasite lines using CRISPR/Cas9. Sci Rep, 2017, 7(1), 3881.

69. Zetsche, B.; Volz, S.E.; Zhang, F. A split-Cas9 architecture for inducible genome editing and transcription modulation. Nat. Biotechnol. 2015, 33, 139–142, https://doi.org/10.1038/nbt.3149.

70. Bork, S.; Okamura, M.; Matsuo, T.; Kumas, S.; Yokoyama, N.; Igashiri, I. Host serum modifies the drug susceptibility of Babesia bovis in vitro. Parasitology 1999, 130, 489−492, https://doi.org/10.1017/s0031182000006821.

71. AbouLaila, M.T., B. Rizk, MA, El-Sayed, SA, Yokoyama, N., Igashiri, I, Serum-free GIT medium for short-term in vitro cultures of Babesia bigemina, Babesia divergens, and Theileria equi. Journal of Protozoology Research, 2017, 27, 13–22.

72. Schrevel, J., P. Grellier, and D. Rigomier, New approaches in in vitro cultures of Plasmodium falciparum and Babesia divergens by using serum-free medium based on human high density lipoproteins. Mem Inst Oswaldo Cruz, 1992, 87 Suppl 3, 71–75.

73. Grande, N.; Precigout, E.; Ancelin, M.-L.; Moubri, K.; Carcy, B.; Lemesre, J.L.; Vial, H.; Gorenflo, A. Continuous in vitro culture of Babesia divergens in a serum-free medium. Parasitology 1997, 115, 81–89, https://doi.org/10.1017/s003118209708937.

74. Martínez, J.A.; Millán, J.V.F.; Ueti, M.W.; Rojas-Martínez, C. Innovative Alternatives for Continuous In Vitro Culture of Babesia bigemina in Medium Free of Components of Animal Origin. Pathogens 2020, 9, 343, https://doi.org/10.3390/pathogens9050343.

75. Kusakasako, K.; Masatani, T.; Yada, Y.; Talactac, M.R.; Hernandez, E.P.; Maeda, H.; Mochizuki, M.; Tanaka, T. Improvement of the cryopreservation method for the Babesia gibsoni parasite by using commercial freezing media. Parasitol. Int. 2016, 65, 532–535, https://doi.org/10.1016/j.parint.2016.02.012.

76. Shikano, S.; Nakada, K.; Hashiguchi, R.; Shimada, T.; Ono, K. A Short Term In vitro Cultivation of Babesia rodhaini and Babesia microti. J. Vet- er- Med. Sci. 1995, 57, 955–957, https://doi.org/10.1292/jvms.57.955.

77. Soga, A.; Bando, H.; Ko-Ketsu, M.; Masuda-Suganuma, H.; Kawazu, S.-I.; Fukushima, S. High efficacy in vitro selection procedure for generating transgenic parasites of Plasmodium berghei using an antibiotic toxic to rodent hosts. Sci. Rep. 2017, 7, 4001, https://doi.org/10.1038/s41598-017-04244-0.

78. Sidik, S.M.; Huet, D.; Ganesan, S.M.; Huynh, M.-H.; Wang, T.; Nasamu, A.; Thiru, P.; Saeij, J.P.; Carruthers, V.B.; Niles, J.C.; et al. A Genome-wide CRISPR Screen in Toxoplasma Identifies Essential Apicomplexan Genes. Cell 2016, 166, 1423–1435.e12, https://doi.org/10.1016/j.cell.2016.08.019.