Establishment of a transient transfection system for *Babesia* sp. Xinjiang with the use of homologous promoters

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Short Report

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Abstract

Background

Babesia species, the agentic pathogens of human and animal babesiosis, are spread worldwide. Over the last decade, genetic manipulation approaches have been applied with many protozoan parasites, including *Plasmodium falciparum*, *Trypanosoma cruzi*, *Cryptosporidium parvum*, *Theileria annulata*, *Theileria parva*, *Babesia bovis*, *Babesia bigemina*, *Babesia ovata*, *Babesia gibsoni*, and *Babesia ovis*. For *Babesia* sp. Xinjiang (*Bxj*), which is the causative pathogen of ovine babesiosis mainly in China, the efficiency of these techniques remain unclear.

Methods

First, a plasmid bearing the elongation factor-1 alpha promoter, as well as the firefly luciferase reporter gene and rap stop region were transfected into *Bxj* by electroporation and nucleoporation to determine the most suitable transfection solution. Then, six program setting were evaluated to confirm the best for *Bxj* transient transfection and a series of different amounts of plasmid DNA were applied to generate relatively high luminescence values. Finally, the activities of four promoters derived from *Bxj* were evaluated using the developed transient transfection system.

Conclusions

In this study, a transient transfection system for *Bxj* was optimized. These findings provided critical information for *Bxj* genetic manipulation, as an essential tool to identify virulence factors and to further elucidate the basic biology of pathogens.

Background

Babesiosis, caused by apicomplexan intraerythrocytic protozoan pathogens of the genus *Babesia* infective to humans, as well as domestic and wild animals, is a re-emerging tick-borne disease in tropical and subtropical regions worldwide [1]. Since the first documented *Babesia* species was reported in 1888, more than 100 others have been identified globally, although few (*Babesia ovis*, *Babesia motasi*, and *Babesia crassa*) have been confirmed as causative agents of ovine babesiosis. This group of *Babesia* spp. has received much attention since *B. ovis* is the main etiological agent of ovine babesiosis, while *B. motasi* and *B. crassa* have been implicated in human cases of babesiosis throughout Asia [2–6]. Additionally, a novel *Babesia* species, named *Babesia* sp. Xinjiang (*Bxj*), experimentally transmitted by *Hyalomma anatolicum anatolicum* (Koch, 1844), was first isolated from a sheep bitten by *Rhipicephalus sanguineus* and *H. anatolicum anatolicum* [7]. Epidemiological investigations revealed that the seropositivity rate of *Bxj* ranged from 0 to 68.13% in 22 provinces across China [8]. Ovine babesiosis is an important socioeconomic factor, particularly in animal mortality, costs of livestock treatment, and productivity loss [9].

The difficulty in controlling ovine babesiosis is due to the lack of effective vaccines, limited choices of therapeutic drugs, and the emergence of several drug-resistant *Babesia* strains. Understanding the basic biology of *Babesia* species is pivotal to the development of effective therapeutic drugs and preventive vaccines to control this disease [10]. Genetic manipulation tools are considered as effective measures to identify virulence factors of particular agents, understand host-parasite interactions, and elucidate the mechanisms underlying drug resistance [11]. Such techniques have been described in several protozoan parasites, including *Plasmodium falciparum*, *Toxoplasma gondii*, *Cryptosporidium parvum*, *Theileria annulata*, *Babesia bovis*, *Babesia bigemina*, *Babesia ovata*, *Babesia gibsoni*, and *Babesia ovis* [12–21]. Mapping and sequencing of the *Bxj* genome were completed by our laboratory (Vector and Vector-Borne Diseases Laboratory, Lanzhou Veterinary Research Institute) in 2006. In this study, a suitable transfection system for *Bxj* merozoites was developed using electroporation and nucleofactor technologies.

Methods

In vitro culture of *Babesia* sp. Xinjiang

A monoclonal line (G5) of *Bxj* was cultured *in vitro* as reported previously [22]. Briefly, when parasitemia reached 20%–30%, 4 mL of a suspension of infected red blood cells (RBCs) were cultured in a T75 flask supplemented with 5% fresh sheep erythrocytes and 20% fetal bovine serum (Gibco, Carlsbad, CA, USA) in Roswell Park Memorial Institute 1640 medium (Lonza Biologics, Portsmouth, NH, USA) at 37°C under an atmosphere of 5% CO₂/95% air.

Plasmid constructs

Schematic diagrams of the transient transfection plasmids used in this study are presented in Fig. 1a and 1b. Five distinct plasmids were generated, named as phosphate-buffered saline (PBS)-LuRaA, PBS-LuRaB, PBS-LuRaAc, and PBS-LuRaRa. Initially, the plasmid PBS-LuRa was constructed by amplifying the *firefly luciferase* gene and *Bxj* rho-try-associated protein-1 3'-flanking region (rap3') by PCR from the plasmid pENT12luc and genomic DNA of *Bxj*, respectively, using specific primer pairs (Table 1). The PCR products were inserted into the *Xho* I restriction site of the plasmid pBluescript SK(+) (provided by Carlos E. Suarez) using the MultiS One Step Cloning Kit (Vazyme Biotech Co., Ltd., Nanjing, China). Then, the promoters of *Bxj* ef1 α IG-A, ef-1 α IG-B, actin 5'-untranslated region (UTR), and rap 5'-UTR were amplified by PCR and cloned into the *Sa*/I restriction site of PBS-LuRa, respectively. A free-promoter plasmid (PBS-LuRa) was used as a negative control. The constructed plasmids were confirmed by sequencing and purified using the QIAGEN Plasmid Maxi Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions.

Optimization of parasite transfection

When parasitemia of *Bxj*-infected RBCs reached 25%–30%, the cultures were centrifuged at 800 × *g* for 10 min. Then, the supernatant was discarded and pellets of *Bxj*-infected RBCs were washed twice in PBS and once in cytomix buffer (120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄/KH₂PO₄, pH 7.6, 25 mM

HEPES, pH 7.6, 2 mM EGTA, 5 mM MgCl₂, final pH 7.6) [14]. Parasite transfections were performed with the use of a Gene Pulser Xcell™ Electroporation System (Bio-Rad Laboratories, Hercules, CA, USA) and 0.2-cm cuvettes containing 50 µL of sterilized cytomix buffer or 20 µg of plasmids and 1 × 10⁸ infected RBCs to a final volume of 100 µL. Initially, two types of nucleofection buffer (human T cell Nucleofector solution and Basic Parasite Nucleofector® Solution) were used to screen the optimization buffer with the program settings U-033, V-024, X-001, D-023, T-023, and T-020. Cytomix buffer was used for electrotransfection at 1200 V and 25 µF. Subsequently, optimized parameters for *Bxj* transfection were employed to determine the amounts of plasmid, which varied from 5 to 150 µg. After transfection, the mixtures were transferred into the wells of a 24-well culture plate containing 7.5% fresh sheep RBCs and 10% fetal bovine serum. The luciferase activities and percentage of parasitized erythrocytes (PPEs) were determined at 24, 48, and 72 h post-transfection. Then, 20 µg of each constructed plasmid (PBS-LuRaA, PBS-LuRaB, PBS-LuRaAc, and PBS-LuRaRa) were transfected into infected RBCs using the optimized *Bxj* transfection conditions. Promoter activity was evaluated with the use of a ONE-Glo™ Luciferase Assay System (Promega Corporation, Madison, WI, USA) at 24, 48, and 72 h post-transfection. Briefly, 100 µL of ONE-Glo™ Luciferase Assay Reagent was mixed with 100 µL of transfected *Bxj* culture in the wells of white 96-well plates and then incubated for 5 min at room temperature. Afterward, the chemiluminescence values were determined at 10-s integration intervals using a GloMax®-Multi Detection System (Promega Corporation).

Results And Discussion

Optimization of *Bxj* merozoite transfection parameters

Experimental conditions for transit transfection of *Bxj* merozoites were determined using a Gene Pulser Xcell™ Electroporation System and a Nucleofector™ II/2b Transfection Device (Lonza). To confirm the suitability of the transient transfection solution, luciferase activity and PPEs were determined at 24, 48, and 72 h, when 20 µg of plasmid DNA with each buffer (human T cell nucleofector solution and Basic parasite nucleofector solution) were transfected with the V-024 program setting, and while the cytomix buffer was electrotransfected with 20 µg of PBS-LuRaB at 1200 V and 25 µF (Fig. 1c). As compared to the other two solutions, the luciferase activity of the transfection system with human T cell nucleofector solution was relatively high (^{***} $p < 0.001$), indicating that this solution was suitable for *Bxj* transient transfection. With the exception of the cytomix buffer, there was no decrease in relative luciferase activity in the human T cell nucleofector solution or basic parasite nucleofector solution over a period of 72 h. In previous reports, there was a downward trend in relative luciferase activity during transfection of *B. bovis*, *B. ovis*, and *Babesia microti*. One possible explanation for this exceptional situation was that human T cell nucleofector solution and basic parasite nucleofector solution were more capable of conveying protective effects to the ovine erythrocyte membrane.

In terms of the impact of each transfection buffer to PPEs, a significant high figures using basic parasite nucleofector solution can be obtained at 72 h post-transfection compared to other transfection solutions (Fig. 1d), while the highest level of relative luminescence units observed in human T cell nucleofector

solution group instead of cytomix and basic parasite nucleofector solution groups (Fig. 1c). These data provide additional evidence that human T cell nucleofector solution was more effective and suitable for transient transfection of *Bxj*.

Furthermore, as compared to the other five program settings (U-033, X-001, D-023, T-023, and T-020), luciferase activity was significantly greater with V-024 ($^{****}p < 0.0001$). Relative luciferase activities at 24, 48, and 72 h post-transfection are presented in Fig. 2e. Subsequently, human T cell Nucleofector solution and V-024 program were used as transient transfection parameters to determine the amount of plasmid DNA. With an increase in the amount of plasmid DNA, relative luminescence units tended to increase, while almost similar values were observed with 20, 50, and 100 μg ($p > 0.05$) (Fig. 1f). As compared to the use of 10 μg , 20 μg generated a relatively high relative light unit value ($^{*}p < 0.05$). Taken together, these results, including relative light units and PPEs, showed that human T-cell solution, the program V-024, and 20 μg of plasmid DNA were the most favorable conditions for transient transfection in subsequent studies.

Evaluation of promoter activity

To determine promoter activity, the optimal transfection conditions mentioned above, including the transfection program, buffer, and amount of plasmid DNA, were employed to transfect *Bxj* merozoites with five types of plasmids (PBS-LuRaA, PBS-LuRaB, PBS-LuRaAc, PBS-LuRaRa and PBS-LuRa). The time courses of luciferase activities in the transfected *Bxj* culture are presented in Fig. 2a. Although four of the promoters tested in this study were able to promote luciferase expression, a significantly higher level of luciferase activity was observed with transfection of the *Bxj* merozoites with either plasmid PBS-LuRaAc or PBS-LuRaB after electroporation. According to values of luciferase activity post-transfection, the extent of promoter activity can be classified as strong (actin 5' UTR), medium (ef1 α IG-B), or low (ef1 α IG-A and rap 5'-UTR). The PPEs of each group at 24, 48, and 72 h post-transfection were determined by calculating the proportion of infected RBCs out of 2000 cells under a light microscope. There were no obvious difference in PPE values among the four groups at 24, 48, and 72 h post-transfection ($p > 0.05$) (Fig. 2b).

Conclusions

In conclusion, the conditions for transient transfection of *Bxj*, including the transfection solution, program settings, and quality of plasmid DNA, were optimized. In addition, promoter activity was systematically evaluated. This study is the first effort to create a platform for transient transfection of *Bxj* and provides basic information for the development of a stable transfection method for *Bxj* and to further elucidate the biology of this parasite, as well as the parasite-vector and parasite-host interactions.

Abbreviations

Bxj: *Babesia* sp. Xinjiang

RBCs: Red blood cells

PCR: Polymerase chain reaction

Declarations

Ethical Approval and Consent to participate

The study was approved by the Animal Ethics Committee of the Lanzhou Veterinary Research Institute, CAAS (Permit No. LVRIAEC-2018-001). All the procedures were conducted according to the Animal Ethics Procedures and Guidelines of the People's Republic of China.

Consent for publication

All authors consent to be published.

Availability of supporting data

All data are available upon request.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

XW and JW carried out the experiments. JW wrote the draft of the manuscript. JL, JY, ZL, AL, YL, YuL and HL participate plasmid construction. GG corrected the manuscript. GL, JLuo and HY supervised all parts of the study. All authors have read and approved the final version of this manuscript.

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Tables

Table 1. The information of primers used in this study

Element	Primers	Sequence (5'-3')	Size (bp)
<i>firefly luciferase</i> gene	<i>luc-F</i>	GGTACCGGGCCCCCCTCGAGatggaagatgccaaaaac	1653
	<i>luc-R</i>	TCGATACCGTCGACCTCGAGttacacggcgatcttgccg	
Promoter	<i>ef1a IG A-F</i>	CGAATTGGGTACCGGGCCCCGATAATACTAGGGTTATTG	897
	<i>ef1a IG A-R</i>	CCATCTCGAGGGGGGGGCCCTTTCGTGGAGTTTTAACTAG	
	<i>ef1a IG B-F</i>	GCGAATTGGGTACCGGGCCCCGAAAGGAAAGGTAACGGC	958
	<i>ef1a IG B-R</i>	CCATCTCGAGGGGGGGGCCCTTTCGTGGAGTTTTAACTAG	
	<i>rap 5' URT-F</i>	GGCGAATTGGGTACCGGGCCCCGAGGATGAGGTATCTCAG	1386
	<i>rap 5' URT-R</i>	CCATCTCGAGGGGGGGGCCCTCTTAACACCAGGCAATGAA	
	<i>actin 5' UTR-F</i>	GGCGAATTGGGTACCGGGCCCAAGTGTCCCAGTTGCCTC	2489
	<i>actin 5' UTR-R</i>	CCATCTCGAGGGGGGGGCCCTTTTCAAAAATTCTGTG	
Terminator	<i>rap3' UTR-F</i>	GCTTATCGATAACGTCGACGCAGCTTCAAAGCGTACAT	1362
	<i>rap3' UTR-R</i>	GGGCCCCCCTCGAGGTCGACTCTTAACACCAGGCAATGA	

Restriction enzyme sites are underlined

Figures

Figure 1

Optimization of the Bxj transient transfection parameters. (a) A diagram of the Bxj *ef-1α* locus structure. (b) The structures of five types of plasmids used for transient transfection. (c) Transfection buffer optimization by electroporation and nucleofection (V-024 program setting). (d) The time course of PPEs was determined after transfection. (e) The optimization of program settings for Bxj transient transfection. (f) Determination of the optimal amount of plasmid DNA for Bxj transfection.

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Figure 2

Promoter activities. (a) Evaluation of promoter activities with optimized transfection conditions. (b) PPEs at 24, 48, and 72 h post-transfection of each type of plasmid DNA bearing different promoters.

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