

Evaluation of the inhibitory effects of drugs on the growth of *Babesia gibsoni* using relative quantification real-time PCR

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Abstract. To evaluate the inhibitory effects of drugs on the growth of *Babesia gibsoni*, relative quantification real-time PCR method was developed in this study. The *18S rRNA* gene was used as a target gene for the $2^{-\Delta\Delta C_t}$ method analysis. Additionally, chicken RNA was added to the parasitized blood before total RNA extraction. The chicken β -actin gene was selected as an internal control gene for the $2^{-\Delta\Delta C_t}$ method analysis. The 100 μ L parasitized blood samples with different percentages of parasitized erythrocytes (PPEs) (3%, 1.5%, 0.75%, 0.375% and 0.1875%) were prepared for relative quantification of *B. gibsoni*. Regression analysis results revealed significant linear relationships between the relative quantification value and parasitemia. *18S rRNA* gene expression was significantly decreased after treatment with diminazene aceturate and artesunate *in vitro* drug sensitivity test. This result suggested that this relative quantification real-time PCR method can be used to evaluate the effects of drug inhibition.

INTRODUCTION

Babesia is transmitted by ticks to vertebrates and results in severe economic losses in the livestock industry worldwide. Human babesiosis is also global problem, and presenting a significant health burden in areas where it is endemic (Tonnetti *et al.*, 2019). *Babesia gibsoni* infection occurs frequently in dogs and has recently become a serious problem because this disease typically results in severe clinical problems (fever, thrombocytopenia, regenerative anemia, splenomegaly, and sometimes death) and recurrent infections even after treatment with antibabesia drugs (Goo & Xuan, 2014). Several antibabesia drugs have been in use for many years, such as diminazene aceturate, imidocarb dipropionate, artesunate, atovaquone, and atovaquone with azithromycin (Goo *et al.*, 2010; Checa *et al.*, 2017; Tuvshintulga *et al.*,

2019). However, most drugs have proven to be ineffective because of problems related to toxicity and the development of resistant parasites (Mosqueda *et al.*, 2012; Checa *et al.*, 2017). Therefore, the development of more effective drugs against *Babesia* with low toxicity to hosts has is desired.

At present, there is no recognized standard in practice for drug screening or activity evaluation. More recently, *in vitro* assays have been proposed to determine the susceptibilities of *Babesia* to drugs (Matsuu *et al.*, 2008; AbouLaila *et al.*, 2010; Silva *et al.*, 2013; Rizk *et al.*, 2017). Parasite-infected red blood cells (RBCs) were diluted with uninfected RBCs to obtain the RBC stock with parasitemia. Then, RBCs with parasitemia were cultured in medium containing the indicated concentration of drugs and incubated at 37°C in a humidified multigas water-jacketed incubator. The sensitivity against drugs was evaluated by measuring

the rate of parasite growth inhibition. The rate of parasitism was calculated by counting the parasitized RBCs to approximately 1000 RBCs in Giemsa-stained thin blood smears. However, microscopic examination of Giemsa-stained thin blood smears requires good-quality smears, and significant differences in parasitemia estimated by different personnel may be found (Rizk *et al.*, 2015).

mRNA has been used as a marker of viability, as it is a highly labile molecule with a very short half-life. Therefore, mRNA can provide a closely correlated indication of viability status. Ribosomal RNA (rRNA) has also been investigated as an indicator of viability (Villarino *et al.*, 2000) and has been found to be positively correlated with viability under some bacterial-killing regimens (McKillip *et al.*, 1998). In this research, we used the *18S rRNA* gene of *B. gibsoni* as a molecular target for relative quantification using real-time PCR.

MATERIALS AND METHODS

Chemical reagents

Diminazene aceturate and artesunate were purchased from Macklin Ltd. (Shanghai, China). Stock solutions of 20 mM were prepared in physiological saline and stored at -20°C until use.

Parasites

B. gibsoni parasites were isolated from a naturally infected dog in Nanjing, China. This parasite was identified as *B. gibsoni* according to 18S rRNA gene sequence analysis (Yao *et al.*, 2014). The blood from the sick dog naturally infected with *B. gibsoni* was kept in liquid nitrogen. Then, this blood was subinoculated into a beagle. Parasites were first detected in blood from the beagle 12 days after inoculation. The blood used in this research was collected from the beagle infected with *B. gibsoni*. Samples were collected in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Jiangsu Province. The protocol was approved

by the Committee on the Ethics of Animal Experiments of Jiangsu Agri-Animal Husbandry Vocational College (20180801).

B. gibsoni relative quantification assay

Parasitized blood was collected from a beagle. Blood smears were made and stained with Wright-Giemsa. The percentage of parasitized erythrocytes was calculated by enumeration of 1,000 total infected and uninfected RBCs on Wright-Giemsa smears at 1,000× under oil using microscopy. Parasitized blood was diluted using uninfected blood collected from normal dog. One hundred microliters of parasitized blood samples with different PPEs (3%, 1.5%, 0.75%, 0.375% and 0.1875%) was used for RNA isolation. Three micrograms of chicken RNA isolated from spleen tissue were added to each blood sample and mixed together before RNA extraction. The total RNA of the mixture was extracted using RNAiso Plus reagent (Takara, Japan) according to the manufacturer's instructions, and the RNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., USA). Reverse transcription-PCR was conducted using a PrimeScript One-Step RT-PCR kit (Takara, Japan) according to the manufacturer's instructions. One primer pair (β -actin F 5'-GAGAATTGTG CGTGACATCA-3' and β -actin R 5'-CCTGAACCTCTCATTGCC A-3') was used to amplify a 157 bp fragment of the chicken β -actin gene (Yao *et al.*, 2016). Another primer pair (B.com 339 F 5'-GTCTT-GTAATTGGAATGATGGTGAC-3' and B.com 339 R 5'-ATGCCCCCAA CCGTTCCTATTA-3') was used to amplify a 339 bp fragment of the *18S rRNA* gene of *Babesia gibsoni* (Yao *et al.*, 2014). Real-time PCR was performed with an Applied Biosystems 7500 Real-time PCR System (Applied Biosystems, USA). The cDNA synthesized from *B. gibsoni* and chicken RNA were diluted continuously to generate calibration curves. PCR amplification efficiencies of the *B. gibsoni* *18S rRNA* gene and chicken β -actin gene were established by means of calibration curves. The expression levels of the *18S rRNA* gene, which indicated the quantity of

B. gibsoni, were analyzed with the $2^{-\Delta\Delta C_t}$ method. Each test was performed in triplicate. The 3% PPE group was identified as the control.

The quantity of *B. gibsoni* was calculated according to Eq. 1.

$$-\Delta\Delta C_T = -[(C_{T \text{ 18SrRNA of } B.g} - C_{T \beta\text{-actin of chicken}}) \text{ test group} - (C_{T \text{ 18SrRNA of } B.g} - C_{T \beta\text{-actin of chicken}}) \text{ control group}] \quad (1)$$

where $C_{T \text{ 18SrRNA of } B.g}$ was the value of the *B. gibsoni* 18S rRNA gene. $C_{T \beta\text{-actin of chicken}}$ was the value of the chicken $\beta\text{-actin}$ gene.

Drug sensitivity test

For the *in vitro* drug sensitivity test, 100 μL parasitized blood with 3% PPE and 200 μL of artesunate (880 nM, 220 nM and 55 nM, respectively) were distributed per well in 24-well plates with three replicates per concentration. These plates were incubated at 37°C for 24 h. RNAiso Plus reagent and 3 μg chicken RNA were added to the well for total RNA extraction. Real-time PCR was used for *B. gibsoni* relative quantification, and the 0 nM group was identified as the control. Different concentrations of diminazene aceturate solution (800 nM, 80 nM and 8 nM) were used for the *in vitro* drug sensitivity test as well.

Statistical analysis

The data in the figure are presented as the arithmetic mean \pm standard deviation (SD). The statistical analysis was performed by one-way analysis of variance (ANOVA) using Predictive Analytics Software 18.0. Duncan's multiple-range test was used, with differences considered to be significant at $P < 0.05$.

RESULTS

B. gibsoni relative quantification assay

The melting curve of the *B. gibsoni* 18S rRNA gene and chicken $\beta\text{-actin}$ gene indicated that the primers used in this study were specific for real-time PCR amplification. The PCR amplification efficiencies of *B. gibsoni* 18S rRNA gene and chicken $\beta\text{-actin}$ gene were

97.7% and 96.7%, respectively. These results suggested that the real-time PCR used in this study was reliable. *B. gibsoni* relative quantification is shown in the Figure 1. The magnitude of linearity ranged from 0.375% to 3% PPE. The equation of the parasitized blood volume versus the *B. gibsoni* relative quantification obtained was $y = 0.323x$ with an R^2 of 0.987. Significant linear relationships between the relative quantification and parasitemia were observed.

Drug sensitivity test for artesunate

The expression levels of the 18S rRNA gene in the artesunate-treated group were downregulated compared to those in the control group ($P < 0.05$). The levels of the 18S rRNA gene were 27% lower at 880 nM artesunate (Figure 2). It was suggested that *B. gibsoni* was significantly suppressed in the presence of 55 nM, 220 nM and 880 nM artesunate. The higher the concentration of artesunate used, the better the inhibitory effects achieved were. Artesunate can dose-dependently inhibit *B. gibsoni* *in vitro*.

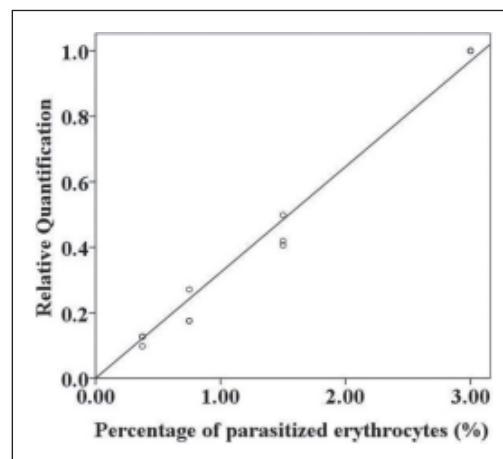


Figure 1. Linearity assessment between *B. gibsoni* relative quantification and percentage of parasitized erythrocytes. Chicken RNA was added to each blood sample before RNA extraction, and the chicken $\beta\text{-actin}$ gene was selected as an internal control. The 18S rRNA gene expression levels of each blood sample with different PPEs (3%, 1.5%, 0.75%, 0.375% and 0.1875%) were analyzed with the $2^{-\Delta\Delta C_t}$ method. The group of 3% PPE was identified as the control.

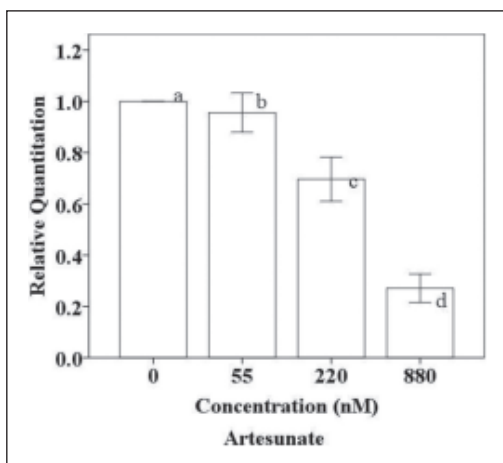


Figure 2. Drug sensitivity test of artesunate. The parasitized blood samples were treated with artesunate (880 nM, 220 nM and 55 nM) and incubated at 37°C for 24 h. The *18S rRNA* gene expression levels of each blood sample were analyzed with the relative quantification real-time PCR method. The 0 nM group was identified as the control. Different letters indicate statistically significant differences ($P < 0.05$).

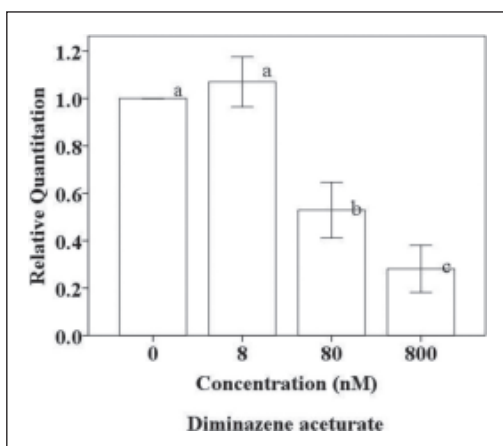


Figure 3. Drug sensitivity test of diminazene aceturate. The parasitized blood samples were treated with diminazene aceturate (800 nM, 80 nM and 8 nM) and incubated at 37°C for 24 h. The *18S rRNA* gene expression levels of each blood sample were analyzed with the relative quantification real-time PCR method. The 0 nM group was identified as the control. Different letters indicate statistically significant differences ($P < 0.05$).

Drug sensitivity test for diminazene aceturate

The expression levels of the *18S rRNA* gene in the diminazene aceturate-treated group (80 nM, 800 nM) were downregulated compared to those in the control group and the 8 nM diminazene aceturate-treated group ($P < 0.05$) (Figure 3). Interestingly, the expression levels were found to be significantly reduced (28%) in the presence of artesunate 800 nM ($P < 0.05$). The lower concentration of diminazene aceturate (8 nM) had no inhibitory effects.

DISCUSSION

In recent years, due to the toxicity and resistance of drugs, the development of new drugs that have a chemotherapeutic effect against babesiosis with high specificity to the parasites and low toxicity to the host is urgently needed. Moreover, the development of a simple *in vitro* system to test the susceptibility of *Babesia* to such drugs is important. Reverse transcription combined with polymerase chain reaction has proven to be a powerful method for quantifying gene expression. Relative quantification real-time PCR describes the change in expression of the target gene relative to a reference group. The method used to present quantitative real-time PCR data was the comparative CT method, also known as the $2^{-\Delta\Delta C_t}$ method. Using this method, the data are presented as the fold change in gene expression normalized to an endogenous reference gene and relative to the untreated control (Livak & Schmittgen, 2001). For the untreated control sample, $\Delta\Delta C_t$ equals zero and 2^0 equals 1, so that the fold change in gene expression relative to the untreated control equals 1, by definition. Housekeeping genes usually suffice as internal control genes and are not affected by experimental treatment. Suitable internal controls for real time quantitative PCR include *GAPDH*, *β -actin*, and *rRNA*.

In this research, the *18S rRNA* gene was used as target gene to quantify the parasites. It has been reported that the mRNA transcripts of the *B. bovis tubulin* beta chain and small subunit rRNA genes are not affected by treatment with lower concentrations of apicoplast-targeting antibacterials (AbouLaila *et al.*, 2012). Due to the instability of mRNA, the nucleic acid degraded when the parasites died. Therefore, mRNA could provide a more closely correlated indication of the viability status of the parasites.

In this research, the *18S rRNA* gene of *B. gibsoni* parasites was selected as the target gene rather than the internal control gene. Because of degradation of mRNA, no gene can be used as an internal control gene. In this study, chicken RNA was added to the parasite RNA and the β -actin gene of chicken was selected as an internal control gene. The same quantity of chicken RNA was added to both the treated group and untreated control group to confirm the same quantification expression of β -actin gene. The quantity of *B. gibsoni* were calculated according to the equation where $-\Delta\Delta C_T = -[(C_{T\ 18SrRNA\ of\ B.g} - C_{T\ \beta\text{-actin\ of\ chicken}}) \text{ test group} - (C_{T\ 18SrRNA\ of\ B.g} - C_{T\ \beta\text{-actin\ of\ chicken}}) \text{ control group}]$.

Significant linear relationships between the relative quantification value and parasitemia were observed using the real-time PCR method. To evaluate the inhibitory effects of the drug, diminazene aceturate and artesunate were added to the parasitized blood. The *18S rRNA* gene expression was decreased relative to that in the untreated control. Both diminazene aceturate and artesunate can dose-dependently inhibit the growth of *B. gibsoni* *in vitro*.

Microscopic examination of Giemsa-stained thin blood smears was widely used in evaluation of inhibitory effects of antibabesia drugs. However, this method was influenced by the quality of the blood smears as well as the skill and experience of experimentators (Rizk *et al.*, 2015). Incorporation of radioactive substances had been a common method in *in vitro* antimalarial drug screening. This method is slowly being replaced by a fluorescence-

based method because the former was constrained by problems with disposing of radioactive materials (Quashie *et al.*, 2006; Rizk *et al.*, 2015). These methods must need *in vitro* cultures of *Babesia* parasites for about 4 days. In this study, relative quantification real-time PCR was used for drug evaluation against *Babesia gibsoni* without continuous culture. The lower parasitemia level can also be detected in real-time PCR method. This assay was easier to perform and was found to be reliable and sensitive.

CONCLUSIONS

In summary, relative quantification real-time PCR for RNA was developed in this study. This method can differentiate live parasites and be used to evaluate inhibitory effects on the growth of *Babesia gibsoni*.

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Conflict of Interests

The authors declare that they have no conflict of interests.

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