Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Establishment and Optimisation of Anti-Babesia microti Drug Efficacy Evaluation Method

Meng Yin
Centers for Disease Control and Prevention
Hao-bing Zhang (zhanghb@nipd.chinacdc.cn)
Centers for Disease Control and Prevention
Yi Tao
Centers for Disease Control and Prevention
Jun-Min Yao
Centers for Disease Control and Prevention
Hua Liu
Centers for Disease Control and Prevention
Htet Htet Win
Ministry of Education
Jia-Xu Chen
Centers for Disease Control and Prevention
Le-Le Huo
Centers for Disease Control and Prevention
Bin Jiang
Centers for Disease Control and Prevention
Yu-Fen Wei
Centers for Disease Control and Prevention
Qi Zheng
Centers for Disease Control and Prevention
Cong-Shan Liu
Centers for Disease Control and Prevention
Jian Xue
Centers for Disease Control and Prevention
Qi-Qi Shi
Centers for Disease Control and Prevention

Methodology

Keywords: Babesia microti, robenidine hydrochloride, quantitative PCR, atovaquone, azithromycin, evaluation of drug efficacy

Posted Date: October 15th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-90833/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Abstract

Background

Babesiosis, an infectious zoonotic parasitic disease that occurs globally, is most commonly caused by *Babesia microti*. For a severe infection, a combination of exchange transfusion and support-therapy is used and, for a mild infection, single antibiotics, or a combination of antibiotics and antiprotozoal drugs, are used for 7–10 days for the treatment of babesiosis; however, as these treatments have problems, a new therapy is needed. Although aetiological and molecular biology methods are often used in drug screening, these methods have disadvantages and there is no standard anti-*B. microti* drug screening method.

Method

This study was consisted of basal test, drug-suppressive test and drug-therapeutic test. BALB/c mice were used as the animal model and robenidine hydrochloride (ROBH) was used as a positive drug. By modifying Peter's 4-day suppression method, immunosuppressive and subpassage tests were conducted, in combination with microscopy and real-time fluorescent quantitative PCR (qPCR), the current anti-*B. microti* drug screening method was optimised. SPSS was used for data analysis.

Result

1) In the basal test, there were significant differences in the erythrocyte infection rate (EIR) and relative value of the target gene ($P = 0.011$ and 0.012, respectively) among days. The blood of infected mice 28, 35, and 37 days post-infection (dpi) could infect healthy second-generation mice, even if no parasites could be observed under a light microscope. Resurgence occurred when the immunity of an infected mouse decreased to a certain extent after using an immunosuppressant.

2) In the drug-suppressive test, no *B. microti* was observed under the light microscope in any ROBH group after drug administration. Significant differences were observed in the EIR and relative value of the target gene (both $P < 0.001$) between the control group and ROBH groups. No *B. microti* was observed after the administration of an immunosuppressant or in second-generation mice in ROBH-treated groups; however, *B. microti* was observed in control and proguanil hydrochloride-treated groups.

3) In the drug-therapeutic test, there were significant differences in the EIR and relative value of the target gene between the 100 mg/kg ROBH groups and the control group ($P = 0.049$ and < 0.001, respectively) but not between ROBH groups and atovaquone-azithromycin group ($P = 0.587$ and 0.418, respectively). None of the second-generation mice in any drug-treated group were infected, whereas all the second-generation mice in the control group were infected; parasites could be observed in the control group from the 2^{nd} day after the administration of an immunosuppressant, several parasites were observed in the atovaquone-azithromycin group from the 10^{th} day after the administration of an immunosuppressant, and no parasites were observed in ROBH groups.

Conclusion

ROBH can be used as a positive drug in anti-*B. microti* drug screening trials. qPCR results can be used as a judgment standard in preliminary screening tests and the combination of immunosuppressive and subpassage experiments can be used to validate drug efficacy.

Background

Babesiosis, a zoonosis usually transmitted by tick bites, occurs globally. *Babesia* species live in the red blood cells of mammals and cause similar symptoms to malaria. Currently, more than 100 species and genotypes of *Babesia* that can infect mammals have been identified worldwide, including seven species can cause babesiosis in humans—*Babesia microti*, *Babesia divergens*, *Babesia bovis*, *Babesia canis*, *Babesia duncani*, *Babesia venatorum* (once named *Babesia sp. EU1*), and a new *Babesia sp.*, *KO1* (similar to *Babesia ovine*) [1,2]. Since the first case of babesiosis in humans was reported in Yugoslavia in 1957 [3], more cases have been reported all over the world, including in the USA, France, Germany, Australia, China, Vietnam, and Myanmar; most cases have been reported in the USA [4], most of which were infected by *B. microti* [5].

Most people infected by *B. microti* show mild symptoms, such as fever and anaemia, and return to normal after 2–4 weeks [6]. If these people do not reach hospital in time, or if the parasite goes undetected, they are likely to become *B. microti*-carriers. In carriers, recurrence occurs in low immune states and the parasite can be transmitted mother-to-child, blood donor to immunodeficient blood transfusion recipient, or organ transplant donor to recipient [7,8].

Exchange transfusion and support-therapy are usually used to treat patients with a severe *B. microti* infection, which is characterised by parasitaemia (≥ 10%), severe haemolysis, and organ failure [9]. In patients who experience more than 3 months of parasitaemia but no symptoms, or mild to moderate infection, combination treatment with antibiotics and antiprotozoal drugs, such as quinine-clindamycin, quinine-azithromycin, and atovaquone-azithromycin combinations, is used for 7–10 days [1,10]. However, these treatments have side effects, relapse can occur, and ‘resistance’ has occurred in patients with immune dysfunction [11]. Therefore, new and more effective treatments need to be explored.

Several approaches are used to determine anti-*B. microti* drug efficacy in drug-screening trials. An aetiological method, observation by microscopy after blood smear staining, is the most commonly used. Mahmoud, using BALB/c mice, conducted *in vivo* anti-*B. microti* drug screening experiments for two drugs [12] and showed that the detection rate of this method is 1000 *B. microti*/mL; however, more smears are needed to improve the detection rate when parasitaemia is not high. Aetiological methods require experts to distinguish parasites; otherwise, parasites are easily omitted or mistakenly identified [13].

Marley, using golden Syrian hamsters (*Mesocricetus auratus*), conducted microscopy combined with subpassage tests and observed that an 8-aminoquinoline, WR238605, has good efficacy [14]. This

method has high accuracy but a long experimental period, large number of test animals, and high requirements for experimenters. Yao conducted preliminary screening experiments on 19 drugs (antibiotics and antiprotozoal drugs) based on this method in BALB/c mice and demonstrated that robenidine hydrochloride (ROBH), a drug used for the prevention of coccidiosis in chickens, has the best efficacy on *B. microti* [15].

Molecular biology methods, such as the original polymerase chain reaction (PCR), nested PCR, and real-time fluorescent quantitative PCR (qPCR), are often used in the diagnosis of protozoan infections. There are advantages and disadvantages to each PCR method. Original PCR has a detection limit of 30 parasites and low sensitivity, accuracy, and specificity [16,17]. The sensitivity of nested PCR is higher, 2.4 parasites/μL, but is easily contaminated because of the two rounds of amplification and false-positive results occur frequently [16]. Because the target gene is in the DNA of the parasite in nested PCR, time is needed to clear all DNA after the parasites are killed; thus, in drug efficacy screening tests, a positive result in nested PCR cannot reflect whether a drug is effective promptly. The sensitivity of real-time qPCR (with SYBR Green) is the same as that of nested PCR but the detection limit of the TaqMan probe is up to 12 parasites/μL. Furthermore, real-time PCR can not only be qualitative but also quantify the target gene by Ct value (threshold cycle) [18,19].

Mohamed established the *Babesia* fluorescence assay (BFA) using SYBR Green I to stain DNA and nested PCR to observe nucleic acid residues in animal organs and determine drug efficacy. However, the specificity of BFA is not good, the dye stains not only parasite DNA but also BALB/c mice and microbial DNA, and the test time is long. Additionally, removing blood from mice tail veins for *in vitro* cultivation allows environmental factors to influence the results. Therefore, the use of BFA is inadequate to determine drug efficacy [20].

Some immunological methods, such as the indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), and immune chromatography test (ICT), are also used in the diagnosis of protozoan infections. IFAT is regularly used to detect *Babesia* and diagnose babesiosis in the USA; its sensitivity and specificity are 88%–96% and 90%–100%, respectively [21]. ELISA is suitable for a large number of serum samples but cross-reactions with other sera occur [22-24]. ICT can detect a specific antibody in the serum and show the result using colour. Additionally, as it is simple, accurate, and intuitive, it is widely used as an on-site test [22]. However, antibodies are produced more than 1 week from infection and a positive serum antibody result does not necessarily represent a current infection. Therefore, immunological methods are not applicable for *B. microti* drug screening in the laboratory [13].

Based on this previous research, in this study, current anti-*B. microti* drug screening methods were optimised using BALB/c mice as the animal model and ROBH as the positive drug. Peter's 4-day suppression method was modified and immunosuppressor resurgence and subpassage tests, with microscopy and real-time fluorescent qPCR, were used to establish an efficient, rapid, and accurate method to evaluate anti-*B. microti* drug efficacy *in vivo*. The results of this study may lay the foundation for the screening of reliable anti-*B. microti* drugs.

Method

1. Establishing *B. microti*-BALB/c mice model

The *B. microti Peabody mjr* strain, ATCC®PRA-99TM, obtained from the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences, was conserved by our institute. Female BALB/c mice, aged 6–8 weeks, were inoculated by an intraperitoneal injection of 200 μL of mice blood that contained 10^7 *B. microti*-infected erythrocytes, as previously described [25]. The number of mice used in basal test, drug-suppressive test and drug-therapeutic test was 153, 252 (36 mice in one group, seven groups in total) and 135 (45 mice in one group, three groups in total) respectively.

2. Staining and microscopic examination

Blood was removed from mice tail tips to prepare thin smears on microscope slides. All smears were air-dried, fixed in absolute methanol, stained with Giemsa stain (Solarbio, Beijing, China), and the parasites were counted under ×1000 magnification using a bright-field microscope (Nikon, Tokyo, Japan). Five thousand erythrocytes were scanned for each sample, the number of the erythrocytes infected with *B. microti* was recorded, and the EIR of each sample was calculated.

3. Nucleic acid testing

Genomic DNA was extracted from erythrocytes in ethylenediaminetetraacetic acid-anticoagulated blood using the DNeasy Blood & Tissue Kit (Qiagen, Shanghai, China). A specific fragment of *B. microti* 18S rRNA was set as the target fragment and the primers were Bab 2 (5′-GTTATAGTTTATTTGATGTTCGTTT-3′) and Bab 3 (5′-AAGCCATGCGATTCGCTAAT-3′), as used in a previous study [26]. The mice actin gene was used as a reference gene and the primers were actin-qF (5′-GTGCTATGTTGCTCTAGACTTCG-3′) and actin-qR (5′-ATGCCACAGGATTCCATACC-3′). Real-time fluorescence qPCR was performed in a 10 μL reaction mixture containing 5 μL of 2×AceQ qPCR SYBR Green Master Mix (Low ROX Premixed), 1 μL of DNA template, 0.2 μL of each primer (10 mM), and 3.2 μL of ddH_2O. The reactions were performed under the following conditions: predegeneration at 95 °C for 5 min, followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C. The conditions for the dissociation curve analysis were 95 °C for 5 min, 60 °C for 60 s, and 95 °C for 15 s. qPCR was repeated three times for each sample. The qPCR reactions were performed using ABI QuantStudio7.

4. Subpassage testing

Six healthy female BALB/c mice, aged 6–8 weeks, were inoculated with anticoagulated blood (200 μL for each) from six infected mice in each group 28 days post-infection (dpi), as described in a previous study [14,15]. Thin blood smears were prepared on microscope slides from 7 dpi to 30 dpi, stained with Giemsa stain, and observed under the microscope, as described in Method 2, for parasites. A negative result was recorded if no parasite was detected.

5. Immunosuppressor testing

A dexamethasone sodium phosphate injection was used as the immunosuppressor, as described previously [27]. Thin blood smears from six mice were prepared on microscope slides 2 days after immunosuppressor injection (200 μL for each). The next steps followed Method 2. A positive result was recorded if parasites were observed.

6. Drug-suppressive and drug-therapeutic tests

Inoculated BALB/c mice (using Method 1) were treated with drug 4 h after infection (0 dpi) in the drug-suppressive test [28] and 10 dpi in the drug-therapeutic test, based on the weight of the mice (0.2 mL/10 g), by intragastric gavage. The drug dosage was prepared according to clinical guidelines and previous study (Table 1).

Table 1. Test drug and dosage in each test

	Test drug	Control
Basal test	/	/
Drug-suppressive test	60mg/kg ROBH*4/10days	5% soluble starch solution*4/10days
	100mg/kg ROBH*4/10days	
	25mg/kg guanatol hydrochloride*4/10days	
Drug-therapeutic test	100mg/kg ROBH*10days	5% soluble starch solution*10days
	195mg/kg atovaquone combined 32.5mg/kg(double dose for the first day) azithromycin *10days	

Three mice in each group were randomly selected as samples at each time point in both the drug-suppressive and drug-therapeutic tests. Thin blood smears were prepared on microscope slides and nucleic acid testing was conducted for each sample.

Six mice were used in subpassage testing to infect six second-generation mice 28 dpi and the immunosuppressor was used for 5 days from 28 dpi.

7. Statisticalanalysis

EIR was evaluated by the percentage of infected erythrocytes. The relative value of the target gene was calculated using the $2^{-\Delta\Delta Ct}$ method and 1 dpi mice from each group were used as a reference. In the basal test, a paired samples *t*-test was used to analyse the mean values of EIR and relative value of the target gene of all samples and a linear correlation analysis was used. Repeated measures analysis of variance (two factors and multi-level) was used to analyse the mean values of EIR and relative value of

the target gene of all samples in both drug-suppressive and drug-therapeutic tests. All data analyses were performed using IBM SPSS software, version 19.0. $P < 0.05$ was considered significant.

Results

1. Basal test

Staining, microscopic examination, and nucleic acid testing were performed daily for 46 days from 0 dpi. Subpassage testing and immunosuppressor testing were conducted 28, 35, and 37 dpi. The immunosuppressor was administered daily for 7 days.

1.1 EIR

All inoculated mice were successfully infected with *B. microti*.

Dynamic EIR changes were observed in the examination of thin blood smears (Fig. 1). The acute period was 7–10 dpi and the highest infection rate was detected 9 dpi, with an average EIR of 37%. Few parasites were detected in the chronic infection stage after 20 dpi, which is in accordance with the research of Xu [25] and Cai [29]. The *P* value was 0.011, which indicated that there were significant differences among daily EIRs.

B. microti was small in the early stage of infection and showed diverse morphology, such as dot, ring, pear, amoeba, rod, and Maltese cross shapes. With the increase in EIR, the infected erythrocyte gradually swelled. Several parasites could be observed in one erythrocyte and dark chromatin was observed in parasites. As *B. microti* escaped from a ruptured erythrocyte, the number of parasites in that erythrocyte decreased and the infected erythrocyte returned to normal. As same as described in Cai's study [29].

1.2 Nucleic acid testing

The change tendency is shown in Fig. 1. The highest relative value of the target gene was observed 9 dpi and the peak stage was 7–10 dpi, which was followed by dynamic changes. Statistical analysis showed significant differences among daily relative values of the target gene ($P = 0.012$).

1.3 Subpassage testing

B. microti was observed in the thin blood smear of all second-generation mice 4 to 15 days after infection by the anticoagulated blood of first-generation mice on 28, 35, and 37 dpi. It indicates although no parasites were observed in the thin smear 28, 35, and 37 dpi, the blood was infectious.

1.4 Immunosuppressor testing

B. microti was observed in the thin blood smear 2 days after the intraperitoneal injection of immunosuppressor 28, 35, and 37 dpi; however, the infection rate was low. The EIR reached 36.2% 7 days after the daily intraperitoneal injection of immunosuppressor and continued to increase, up to 61.1%. This

indicated that *B. microti* would appear in the blood again when the immunity of the infected mice in the chronic stage decreases.

2. Drug-suppressive test

All inoculated mice were successfully infected with *B. microti*. Both the examination time point and number of mice in one group were reduced based on the results from the basal experiment, and 4, 7, 11, 15, 20, and 28 dpi were set as examination time points.

2.1 EIR

Blood was extracted from mice tail tips to prepare thin smears on microscope slides at each time point. No parasite was observed in the ROBH group (EIR = 0.00%). The morphology of red blood cells and *B. microti* in ROBH and control groups after infection under a transmission electron microscope is shown in Fig. 2. There was no *B. microti* in mice red blood cells in the ROBH group and the shape of red blood cells tended to be more normal than that in the control group.

There were significant differences between the ROBH and control groups, the ROBH and chloroguanide hydrochloride groups (both $P < 0.001$). The EIR in the control and chloroguanide hydrochloride groups showed dynamic changes and the peak stage was 7–11 dpi (Fig. 3). There were no significant differences between the control group and the 4- and 10-day chloroguanide hydrochloride group ($P = 0.334$ and 0.950, respectively).

2.2 Nucleic acid testing

The relative value of the target gene was < 1.00 in the ROBH group. Significant differences were observed between the ROBH and control groups and the ROBH and chloroguanide hydrochloride groups (both $P < 0.001$). Additionally, there were no significant differences between 4-and 10-day ROBH treated groups for both 100 mg/kg and 60 mg/kg doses. The relative value of the target gene in the chloroguanide hydrochloride and control groups was highest on 7 dpi and 11 dpi, respectively, and declined gradually to < 0.10 from 28 dpi. There were significant differences between the chloroguanide hydrochloride and control groups ($P < 0.001$) and the 4- and 10-day treated chloroguanide hydrochloride groups ($P < 0.001$).

2.3 Subpassage testing

B. microti was observed in the thin blood smear of second-generation mice in both chloroguanide hydrochloride and control groups 7 to 15 days after infection by the anticoagulated blood of first-generation mice 28 dpi. No parasites were observed in the thin smear of second-generation mice in all ROBH groups.

2.4 Immunosuppressor testing

Thin blood smears of mice in each group were prepared 3–30 dpi and *B. microti* was observed in both chloroguanide hydrochloride and control groups but not in the ROBH group. The results of repeated

experiments were consistent. This indicated that, after infected mice in the chronic stage are treated by ROBH, *B. microti* would not appear in blood again.

3.Drug-therapeutic test

The combination of atovaquone and azithromycin is most commonly used for the treatment of patients with *B. microti* infection and was used as a reference to study the efficacy of ROBH. Mice were treated with the drug daily for 10 days from 10 dpi and blood was inspected 10, 13, 15, 18, 21, 24, 28, 31, and 56 dpi. All inoculated mice were successfully infected with *B. microti*.

3.1 EIR

Dynamic changes in EIR are shown in Fig. 4. The EIR reduced to 0.00%, 0.33%, and 0.70% in the ROBH, atovaquone-azithromycin combination, and control groups, respectively, 13 dpi. The *P* value was 0.049, which indicated that there was a significant difference between 100 mg/kg ROBH and control groups. However, there were no significant differences between the 100 mg/kg ROBH and atovaquone-azithromycin combination groups and the control and atovaquone-azithromycin combination groups ($P = 0.587$ and 0.108, respectively).

3.2 Nucleic acid testing

The relative value of the target gene was < 0.10 in the ROBH group from 21 dpi, > 0.10 at any examination time point in the atovaquone-azithromycin combination group, and > 0.30 until 31 dpi and > 0.10 until 56 dpi in the control group. There were significant differences between the 100 mg/kg ROBH and control groups and the atovaquone-azithromycin combination and control groups (both $P < 0.001$). However, there was no significant difference between the 100 mg/kg ROBH and atovaquone-azithromycin combination groups.

3.3 Subpassage testing

B. microti was only observed in the thin blood smear of second-generation mice in the control group.

3.4 Immunosuppressor testing

Thin blood smears of mice in each group were prepared 3–30 dpi and *B. microti* was observed in both atovaquone-azithromycin combination and control groups but not in the ROBH group. Only a small number of *B. microti* was observed in the atovaquone-azithromycin combination group after 10 days of immunosuppressor administration.

Discussion

The aim of this study was to evaluate anti-*Babesia* drug efficacy evaluation methods. Three tests were carried out: basal test, drug-suppressive test, and drug-therapeutic test. The difference between drug-suppressive and drug-therapeutic tests was the timing of drug administration. In the drug-suppressive test, the drug was administered daily for 4 or 10 days from 4 h after inoculation, whereas in the drug-therapeutic test, the drug was administered daily for 10 days from the 10^{th} day after inoculation, when parasitaemia was more acute. To examine the results of all three tests, thin blood smear staining and microscopic examination, nucleic acid testing, subpassage testing, and immunosuppressor testing were conducted.

1.Basal test

Each method verified the results of the others. The trends of EIR and relative value of the target gene in qPCR were consistent, the result of the correlation analysis was significant (Fig. 5; Pearson r = 0.8313, R^2 = 0.6910), and the peak stage based on both measurements was 7–10 dpi. When the EIR was 0.00% after 20 dpi, although it was difficult to confirm infection by microscopy, nucleic acid testing showed a positive result. Simultaneously, second-generation mice were infected, which indicated that infected mice in the chronic stage were infectious and that *B. microti* would recur when infected mice in the chronic stage entered a weakened immune state.

2.Drug-suppressive test

In this test, Peter's 4-day suppression method was modified. Considering the period of clinical therapy, the treatment time was prolonged, and regardless of ROBH dose and treatment time, the EIR was 0.00% from 4 dpi. The relative value of the 18S rRNA encoding gene was < 1.00. Both subpassage and immunosuppressor showed negative results. The results of the control group were similar to those of the basal test.

We also studied the efficacy of chloroguanide hydrochloride, which has a similar structure to ROBH, in the drug-suppressive test. The recommended dosage for chloroguanide hydrochloride is 40 mg/kg but this was toxic and lead to mice death. Therefore, the dosage was gradually lowered to 25 mg/kg. The result showed that, despite its guanidino structure, chloroguanide hydrochloride could not inhibit *B. microti*, which is concordant with the results of our previous study [15].

3.Drug-therapeutic test

Generally, patients enter a hospital when symptoms appear. However, symptoms show after the incubation period of *B. microti*, which can be 1–4 weeks after tick bites and 1–9 weeks after blood transfusion [6]. We treated mice for 10 days from the day the EIR reaches its peak stage, and used the common combination therapy of atovaquone and azithromycin as a reference, to investigate the efficacy of ROBH and verify the accuracy of the method we developed.

The combination of atovaquone and azithromycin relieved parasitaemia quickly but recrudescence appeared in immunosuppressor testing, whereas the ROBH-treated group showed negative results in all four methods. This indicates that the efficacy of the atovaquone and azithromycin combination may be inferior to that of ROBH.

We evaluated these four *B. microti*-detecting methods in their ability to evaluate drug efficacy and observed that thin blood smear staining and microscopy was most intuitive, although it missed parasites easily and required specialists. Additionally, it was difficult to determine whether all parasites died or were simply not detected when we failed to identify *B. microti* by microscopy. Subpassage testing and immunosuppressor testing had high accuracy but a long time and many animals were required. qPCR had high accuracy and could process many samples at one time. The relative value of the target gene was calculated using the $2^{-\Delta\Delta Ct}$ method; thus, both qualitative and quantitative results could be offered by qPCR.

Additionally, nested PCR was used in drug efficacy evaluation in this study (data not shown) but always showed a positive outcome in three tests from 1 dpi to the end of the test. This result is in accordance with those by Xu [26]. Because the elimination of parasite DNA takes time, nested PCR, which provides qualitative results only, could not reflect drug efficacy promptly.

Consequently, we suggest that ROBH is used as a positive drug in anti-*Babesia* drug screening experiments. Methodologically, the modified Peter's 4-day suppression method could be used in preliminary screening and qPCR could be conducted to detect mice blood 4–11 dpi and, if the relative value of the target gene is < 0.10, the tested drug can be considered effective in the inhibition of parasite growth. Then, a drug-therapeutic test could be carried out, in which subpassage testing and immunosuppressor testing could be conducted 28 dpi. If both show negative results, the tested drug can be assumed to be effective for the treatment of babesiosis.

Conclusion

In our study, an anti-*B. microti* drug efficacy evaluation method was established and optimised. ROBH can be used as a positive drug in anti-*B. microti* drug screening trials in the laboratory. The time and number of animals required can be reduced using qPCR as a judgment standard in anti-*B. microti* drug preliminary screening tests. Finally, the combination of immunosuppressive and subpassage experiments can be used to validate drug efficacy.

Abbreviations

ROBH: Robenidine hydrochloride

qPCR : real-time fluorescent quantitative PCR

Ct : threshold cycle

IFAT: fluorescent antibody test

ELISA : enzyme-linked immunosorbent assay

ICT: immune chromatography test

EIR : erythrocyte infected rate

dpi: days post-infection

RBC: red blood cells

Declarations

Ethics approval and consent to participate

Animals were handled in accordance with good animal practice strictly according to the Animal Ethics Procedures and Guidelines of the People's Republic of China. The protocol for sampling from animals had been approved by the Animal Welfare& Ethics Committee of the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention in Shanghai (Permit No:IPD-2019-13).

Consent for publication

Not applicable.

Availability of data and material

The datasets supporting the conclusions of this article are included within the article.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements and Funding

We gratefully thank Shao-Hong Chen,Yu-Chun Cai and Peng Song for valuable suggestions.

This work was supported by the following grants: technology development research for science research institute (2014DFA31130)and the funding scheme for college young teachers of Shanghai Municipal Education Commission (ZZJKYX15007).

Author information

Affiliations

[1]National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, WHO Collaborating Center for Tropical Diseases; National Center for International Research on Tropical Diseases, Ministry of Science and Technology; Key Laboratory of Parasite and Vector Biology.

[2]Pharmaceutical Research Laboratory, Biotechnology Research Department, Ministry of Education, Tasoe Road, Mandalay Region, Kyaukse 05151, Myanmar.

Contributions

Conceived and designed the experiments: HBZ and MY. Performed the experiments: MY, YT,HL ,JMY and HHW. Analyzed the data: MY. Contributed reagents/materials/analysis tools: MY,YT, JMY, JXC, LLH, BJ, YFW, QZ, CSL, JX and QQS. Wrote the paper: MY, HBZ and HHW. All authors read and approved the final manuscript.

Corresponding author

Correspondence to Hao-Bing Zhang. E-mail address: zhanghb@nipd.chinacdc.cn

References

1. Rozej-Bielicka W, Stypulkowska-Misiurewicz H, Golab E. Human babesiosis. Przegl Epidemiol. 2015;69:489–94, 605–608.
2. Kim JY, Cho SH, Joo HN, Tsuji M, Cho SR, Park IJ, Chung GT, Ju JW, Cheun HI, Lee HW, Lee YH, Kim TS. First case of human babesiosis in Korea: Detection and characterization of a novel type of Babesia sp. (KO1) similar to ovine babesia. J CLIN MICROBIOL. 2007;45:2084–7.
3. SKRABALO Z, DEANOVIC Z. Piroplasmosis in man; Report of a case. Doc Med Geogr Trop. 1957;9:11–6.
4. Krause PJ. Human babesiosis. INT J PARASITOL. 2019;49:165–74.
5. Gray EB, Herwaldt BL. Babesiosis Surveillance - United States, 2011–2015. MMWR Surveill Summ. 2019;68:1–11.
6. Vannier E, Krause PJ. Human babesiosis. N Engl J Med. 2012;366:2397–407.
7. Ruebush TN. Human babesiosis in North America. Trans R Soc Trop Med Hyg. 1980;74:149–52.
8. Herwaldt BL, Neitzel DF, Gorlin JB, Jensen KA, Perry EH, Peglow WR, Slemenda SB, Won KY, Nace EK, Pieniazek NJ, Wilson M. Transmission of Babesia microti in Minnesota through four blood donations from the same donor over a 6-month period. TRANSFUSION. 2002;42:1154–8.
9. Wormser GP, Dattwyler RJ, Shapiro ED, Halperin JJ, Steere AC, Klempner MS, Krause PJ, Bakken JS, Strle F, Stanek G, Bockenstedt L, Fish D, Dumler JS, Nadelman RB. The clinical assessment, treatment, and prevention of lyme disease, human granulocytic anaplasmosis, and babesiosis: Clinical practice guidelines by the Infectious Diseases Society of America. CLIN INFECT DIS. 2006;43:1089–134.

10. Shih CM, Wang CC. Ability of azithromycin in combination with quinine for the elimination of babesial infection in humans. AM J TROP MED HYG. 1998;59:509–12.
11. Wormser GP, Prasad A, Neuhaus E, Joshi S, Nowakowski J, Nelson J, Mittleman A, Aguero-Rosenfeld M, Topal J, Krause PJ. Emergence of resistance to azithromycin-atovaquone in immunocompromised patients with Babesia microti infection. CLIN INFECT DIS. 2010;50:381–6.
12. AbouLaila M, Sivakumar T, Yokoyama N, Igarashi I. Inhibitory effect of terpene nerolidol on the growth of Babesia parasites. PARASITOL INT. 2010;59:278–82.
13. Zhang J, Chen J. Current advances in detecting techniques *Babesia microti* infection. Chinese Journal of Zoonoses. 2013;29:714–8.
14. Marley SE, Eberhard ML, Steurer FJ, Ellis WL, McGreevy PB, Ruebush TN. Evaluation of selected antiprotozoal drugs in the Babesia microti-hamster model. Antimicrob Agents Chemother. 1997;41:91–4.
15. Yao J, Zhang H, Liu C, Tao Y, Yin M. Inhibitory effects of 19 antiprotozoal drugs and antibiotics on Babesia microti infection in BALB/c mice. J INFECT DEV COUNTR. 2015;9:1004.
16. Persing DH, Mathiesen D, Marshall WF, Telford SR, Spielman A, Thomford JW, Conrad PA. Detection of Babesia microti by polymerase chain reaction. J CLIN MICROBIOL. 1992;30:2097–103.
17. Caccio S, Camma C, Onuma M, Severini C. The beta-tubulin gene of Babesia and Theileria parasites is an informative marker for species discrimination. INT J PARASITOL. 2000;30:1181–5.
18. Teal AE, Habura A, Ennis J, Keithly JS, Madison-Antenucci S. A new real-time PCR assay for improved detection of the parasite Babesia microti. J CLIN MICROBIOL. 2012;50:903–8.
19. Ohmori S, Kawai A, Takada N, Saito-Ito A. Development of real-time PCR assay for differential detection and quantification for multiple Babesia microti-genotypes. PARASITOL INT. 2011;60:403–9.
20. Rizk MA, El-Sayed SAE, AbouLaila M, Eltaysh R, Yokoyama N, Igarashi I. Performance and consistency of a fluorescence-based high-throughput screening assay for use in Babesia drug screening in mice. SCI REP-UK 2017;7.
21. Krause PJ, Telford SR, Ryan R, Conrad PA, Wilson M, Thomford JW, Spielman A. Diagnosis of babesiosis: Evaluation of a serologic test for the detection of Babesia microti antibody. J INFECT DIS. 1994;169:923–6.
22. Luo Y, Jia H, Terkawi MA, Goo YK, Kawano S, Ooka H, Li Y, Yu L, Cao S, Yamagishi J, Fujisaki K, Nishikawa Y, Saito-Ito A, Igarashi I, Xuan X. Identification and characterization of a novel secreted antigen 1 of Babesia microti and evaluation of its potential use in enzyme-linked immunosorbent assay and immunochromatographic test. PARASITOL INT. 2011;60:119–25.
23. Luo Y, Terkawi MA, Jia H, Aboge GO, Goo YK, Cao S, Li Y, Yu L, Ooka H, Kamyingkird K, Masatani T, Zhang S, Nishikawa Y, Igarashi I, Xuan X. A double antibody sandwich enzyme-linked immunosorbent assay for detection of secreted antigen 1 of Babesia microti using hamster model. EXP PARASITOL. 2012;130:178–82.

24. Priest JW, Moss DM, Won K, Todd CW, Henderson L, Jones CC, Wilson M. Multiplex assay detection of immunoglobulin G antibodies that recognize Babesia microti antigens. CLIN VACCINE IMMUNOL. 2012;19:1539–48.
25. Xu S, Bian R, Chen X: Pharmacological experiment methodology, ed 3. Beijing, People's Medical Publishing House, 2002.
26. Xu B, Liu XF, Cai YC, Huang JL, Zhang RX, Chen JH, Cheng XJ, Zhou X, Xu XN, Zhou Y, Zhang T, Chen SB, Li J, Wu QF, Sun CS, Fu YF, Chen JX, Zhou XN, Hu W. Screening for biomarkers reflecting the progression of Babesia microti infection. Parasit Vectors. 2018;11:379.
27. Lu Y, Cai Y, Chen S, Chen J, Guo J, Chen M, Ai L, Chu Y, Chen Z, Zhou X. Establishment of the Experimental Animal Model of Babesia microti. Chinese Journal of Parasitology Parasitic Diseases. 2012;30:423–7.
28. Peters W. The chemotherapy of rodent malaria, XXII. The value of drug-resistant strains of P. Berghei in screening for blood schizontocidal activity. Ann Trop Med Parasitol. 1975;69:155–71.
29. Cai Y, Lu Y, Chen S, Guo J, Chen M, Tian L, Zhang L, Zhou Y, Chen J. Infection dynamics of *Babesia microti* in mice in different immune status. Chinese journal of Zoonoses. 2013;29:170–4.

Figures

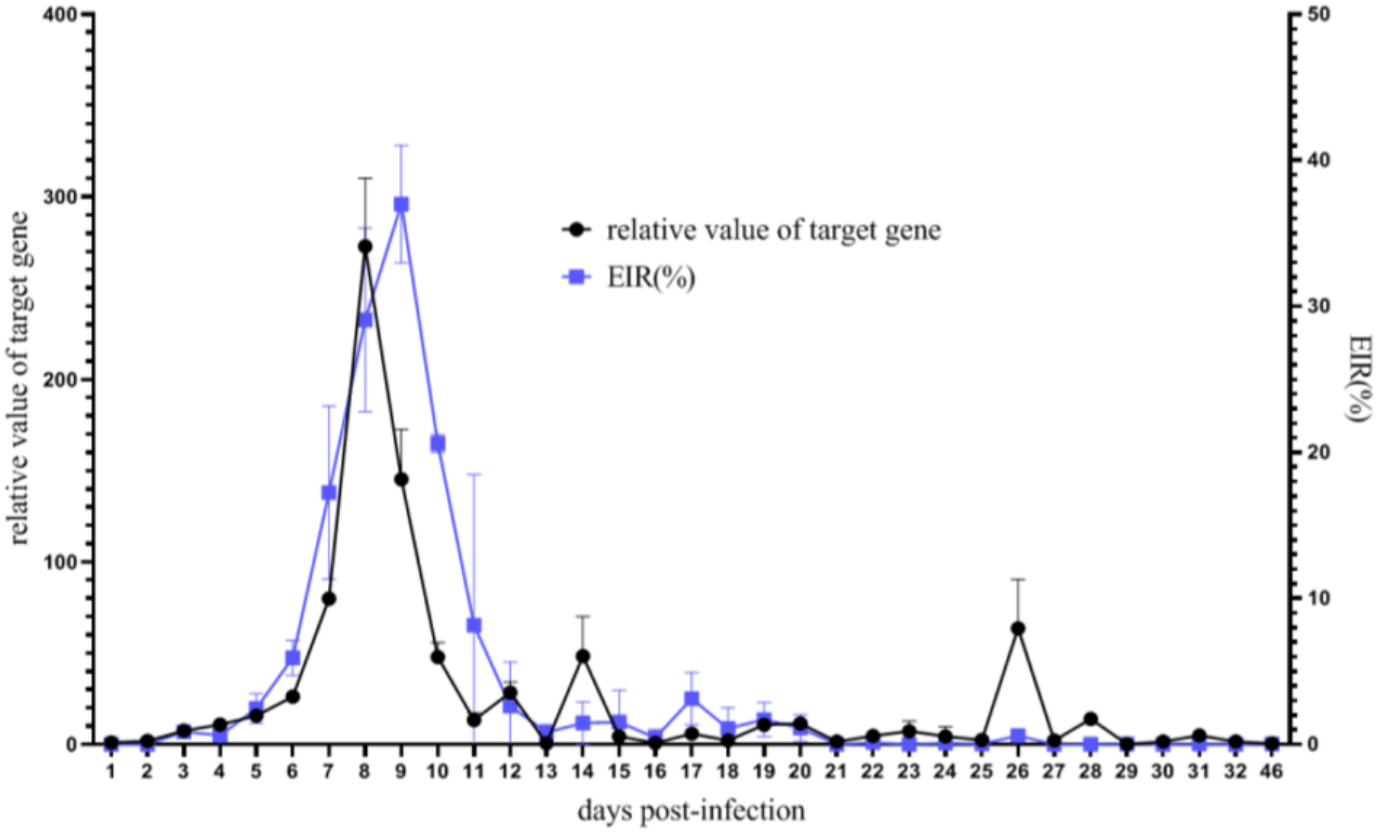


Figure 1

ynamics EIR and relative value of target gene in B.microti infected mice in basal test. Each point represents the mean ± SD

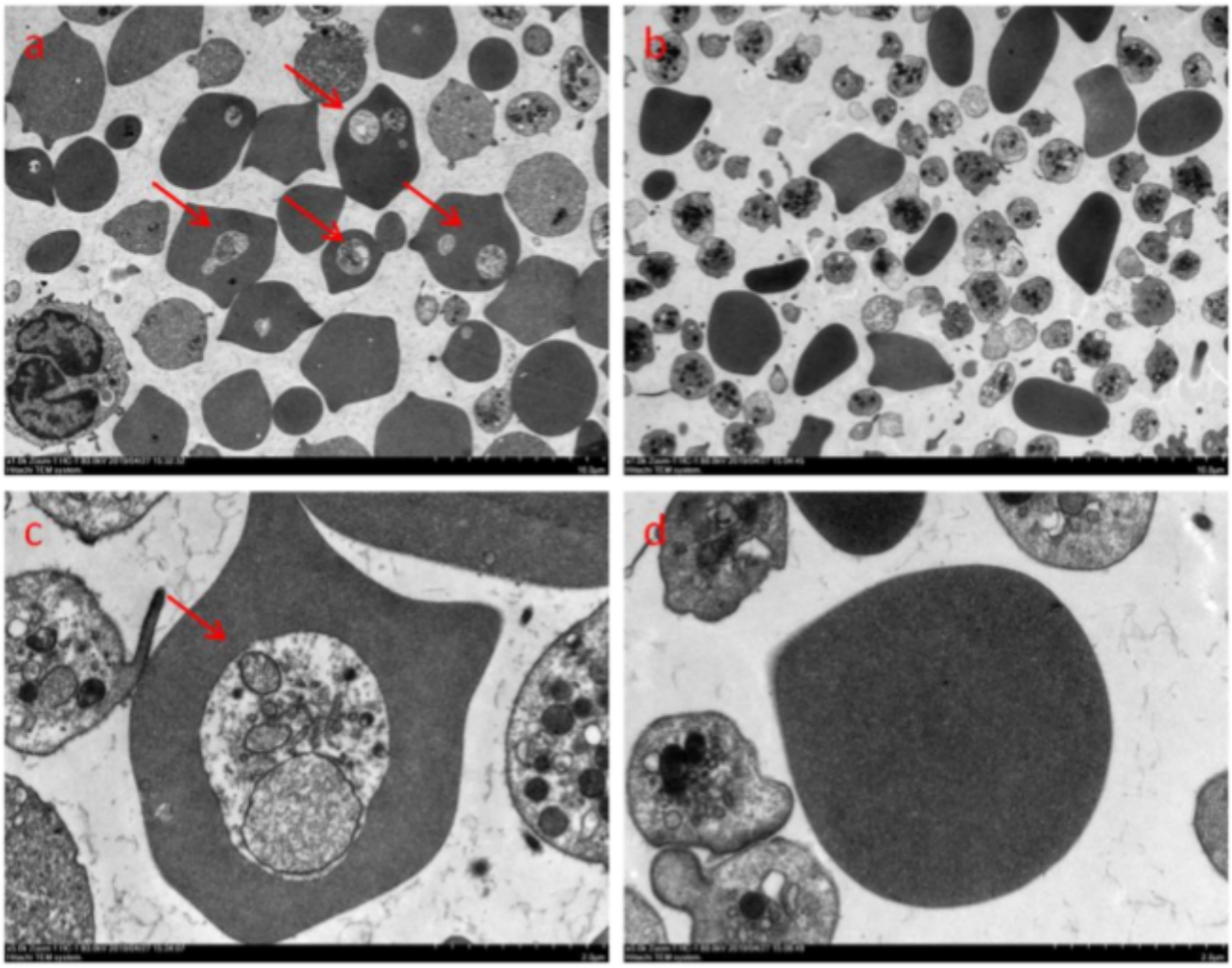


Figure 2

Morphology of red blood cells and B.microti in ROBH and control groups on 11dpi a and c showed morphology of RBC and B.microti in control group, b and d were ROBH group.

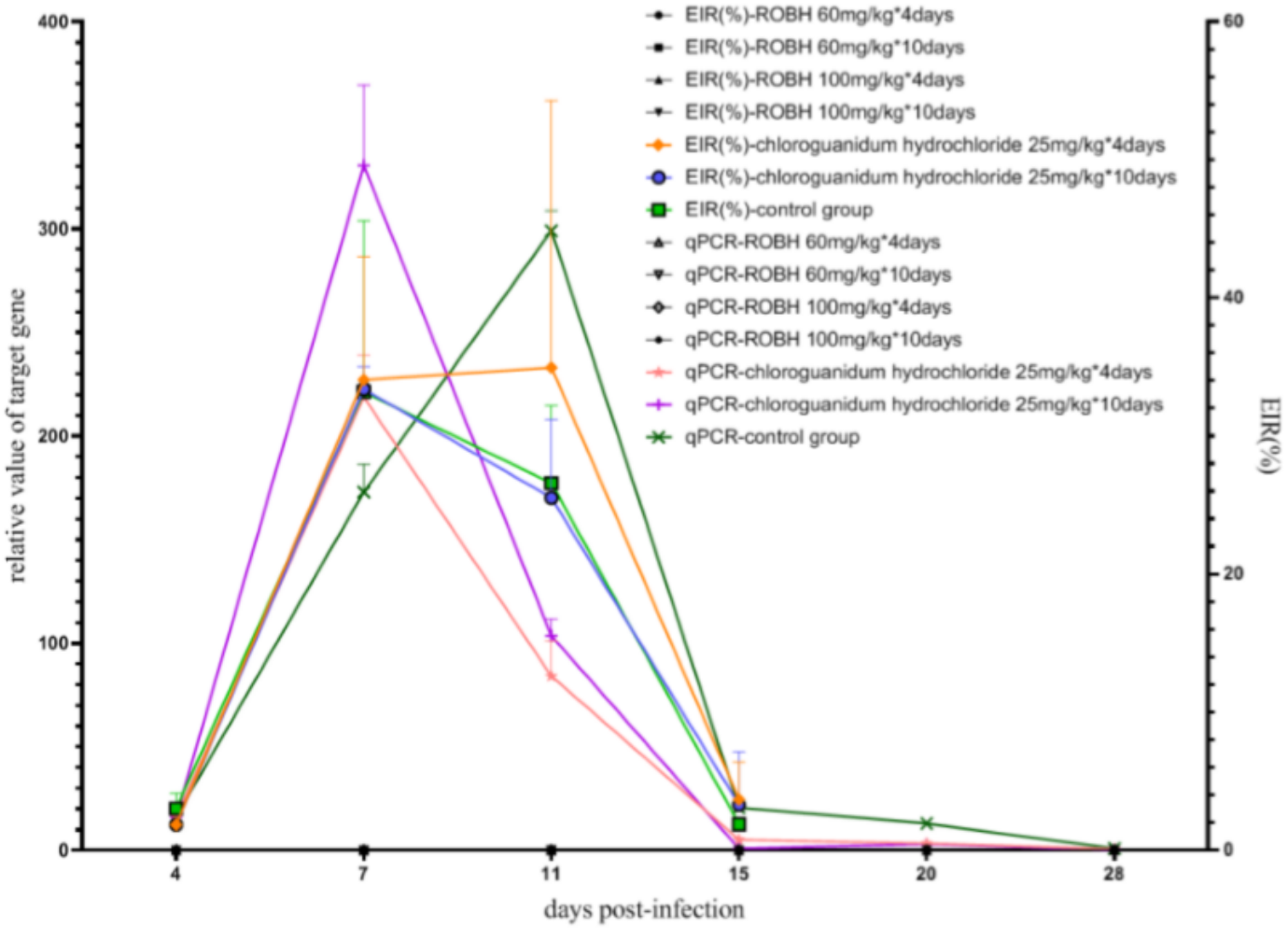


Figure 3

Dynamics EIR and relative value of target gene in B.microti infected mice in drug-suppressive test. Each point represents the mean ± SD

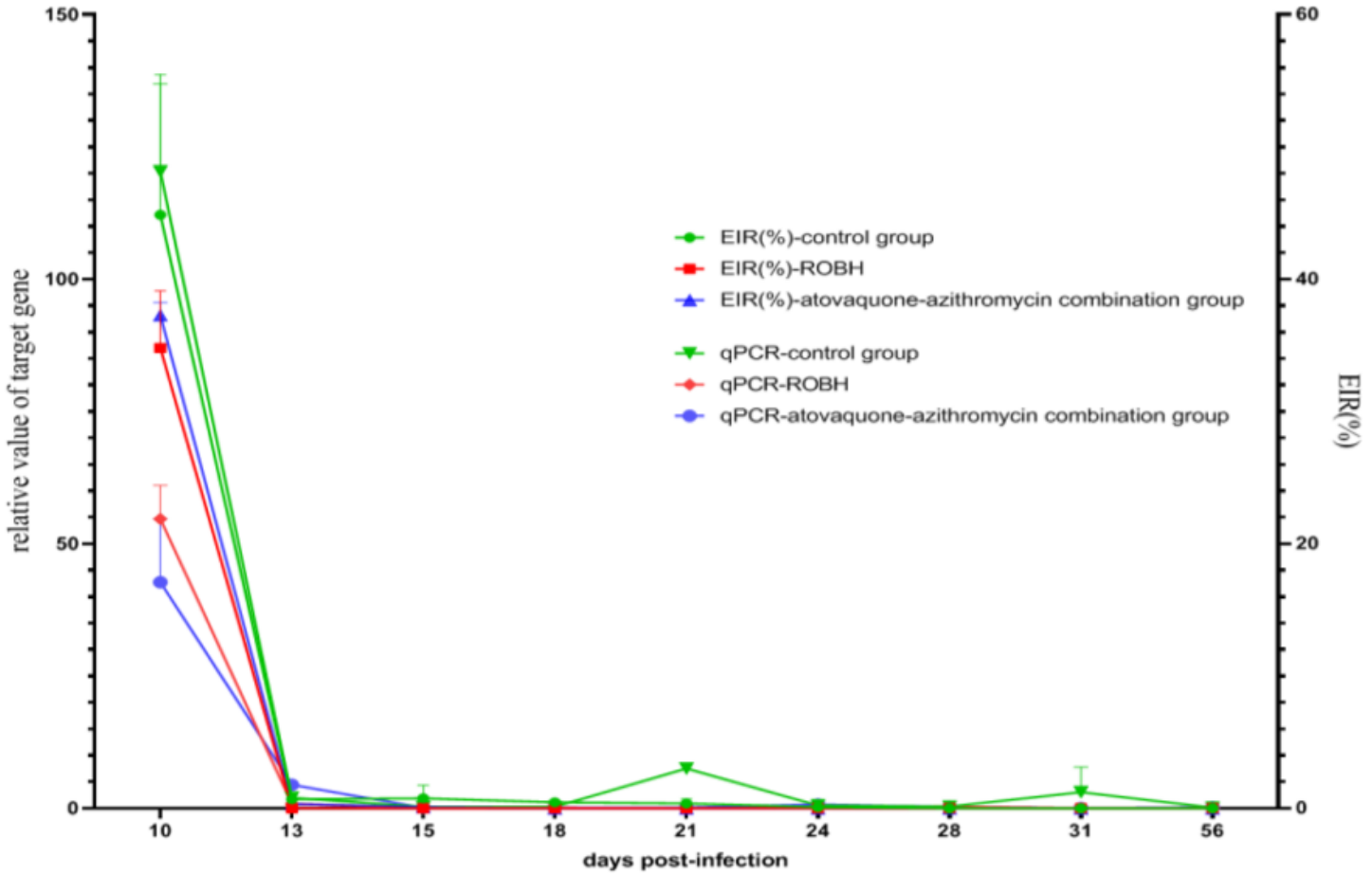


Figure 4

Dynamics EIR and relative value of target gene in B.microti infected mice in drug-therapeutic test. Each point represents the mean ± SD

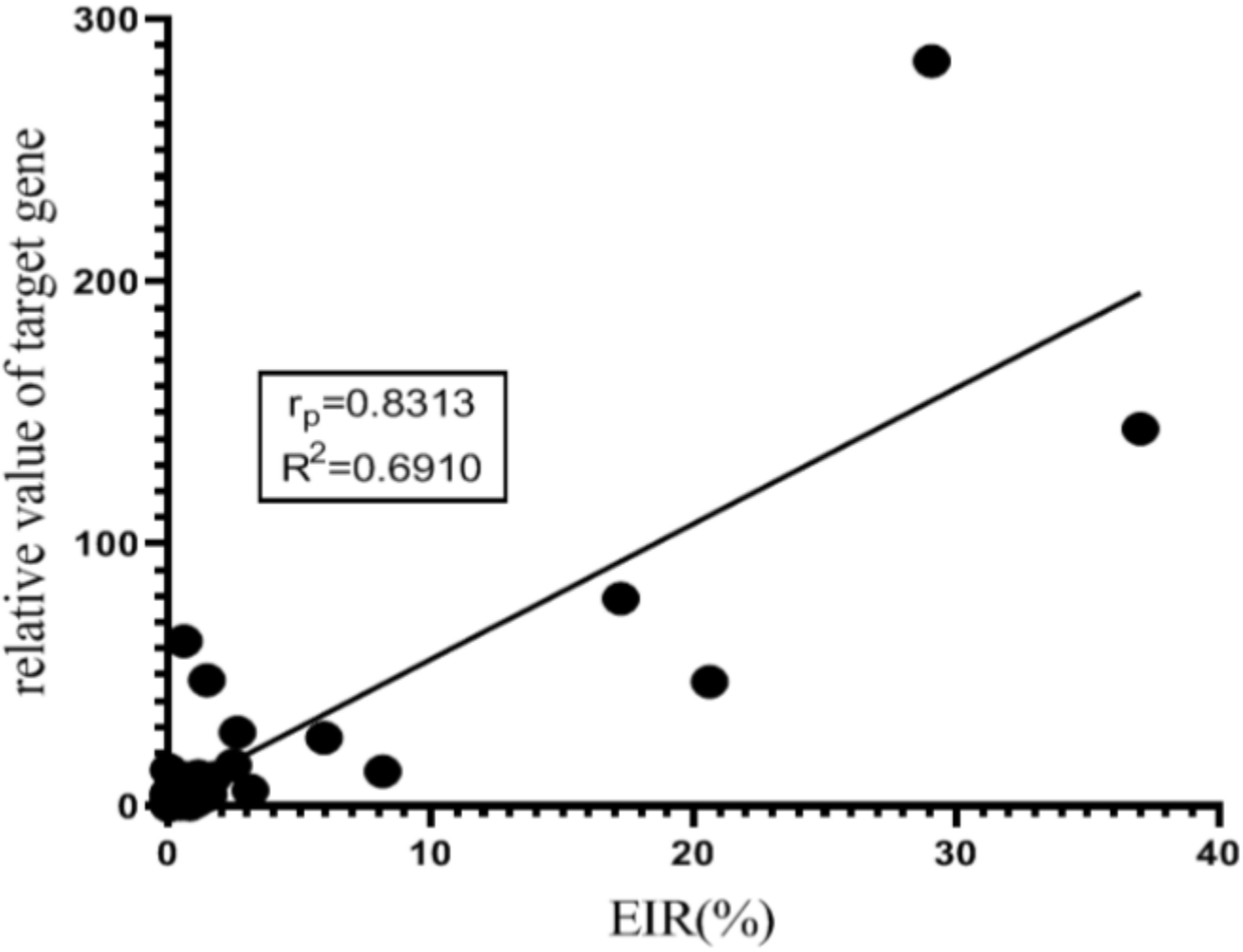


Figure 5

The correlation analysis of everyday EIR and relative value of target gene in basal test