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Evaluation of inhibitory effect of methylene blue against *Babesia* and *Theileria* parasites

Tuvshintulga, B., Sivakumar, T., Salama, A.A., Yokoyama, N. and Igarashi, I.*

*Corresponding Author: Igarashi, I., E-mail: igarcpmi@obihiro.ac.jp

National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan

ABSTRACT

Methylene blue, the first fully synthetic drug, has been widely used in medical treatments. A few decades ago, this drug was used as an antimalarial agent. In this study, the *in vitro* inhibitory effect of methylene blue on *Babesia bovis*, *B. bigemina*, *B. caballi*, and *Theileria equi* (*B. equi*) and the *in vivo* inhibitory effect on *B. microti* were evaluated. Methylene blue significantly inhibited the growth of *B. bovis*, *B. caballi*, and *T. equi* at a 0.1 µM concentration, while *B. bigemina* was significantly inhibited at 0.01 µM, on day 3 of cultivation. The half maximal inhibitory concentrations (IC_{50}) of methylene blue against *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* were calculated as 0.83±0.02, 0.68±0.09, 0.54±0.14, and 0.49±0.06 µM, respectively. The subsequent viability assays, in which drug-free media were used for the cultivation, showed that there were no growths of *B. bovis* or *B. bigemina* that had been previously treated with 10 µM methylene blue. Similarly, *B. caballi* and *T. equi* that had been previously treated with 1 µM methylene blue failed to grow in the viability tests. As for the *in vivo* inhibition assay, the high dose of methylene blue showed a low inhibitory effect on the *in vivo* growth of *B. microti* at 50 mg/kg body weight treatment groups as compared with the untreated group. Therefore, methylene blue might not be used for against *Babesia* and *Theileria* parasites.

Keywords: methylene blue; *Babesia*; *Theileria*; *in vitro*; *in vivo*

INTRODUCTION

Piroplasmosis is a tick-borne intraerythrocytic protozoan disease caused by *Babesia* and *Theileria* parasites. Among the *Babesia* parasites, *B. bovis* and *B. bigemina* are considered to be the most virulent species that infect cattle. On the other hand, *B. caballi* and *T. equi* (before the *B. equi*) are associated with severe clinical piroplasmosis in horses. The transmission of *piroplasms* occurs when the Ixodid ticks that were infected with these parasites feed on the host animals. Clinical signs of bovine and equine piroplasmosis include fever, anemia, jaundice, and hemoglobinuria (Ristic, 1981; Freidhoff, 1988; Schein, 1988). Cattle farming operations suffer

huge economic losses, especially in tropical and subtropical regions, due to this disease (Schein, 1988; McCosker, 1981). There are both direct and indirect economic impacts on livestock farming: the direct impact is mainly due to loss of production, treatment cost, and death of animals; indirectly, expenses such as costs for tick-control strategies add to the financial burden of piroplasmosis (Tisdell *et al*., 1999). Therefore, control of bovine and equine piroplasmosis is of paramount importance for sustainable cattle and horse farming. Tick control, vaccination, and chemotherapy are some widely practiced control methods. Although a systematic application of acaricides might reduce tick infestation and hence the incidence of piroplasmosis significantly reduced, the development of acaricide resistance is of great concern (Kunz and Kemp, 1994). Despite vigorous research works to develop a sub-unit vaccine, only live-attenuated vaccines are currently available for the control of bovine babesiosis caused by *B. bovis* and *B. bigemina* (Bock *et al*., 2004). However, the global use of these vaccines is limited for various reasons, such as vaccine breakthroughs and transmission of other virulent subpopulations that could have contaminated the vaccine strain (Timms *et al*., 1990). Therefore, chemotherapy plays an important role in the control of piroplasmosis in cattle and horses. Very few drugs are available for the treatment of babesiosis and theileriosis, and the toxic side effects associated with the use of these drugs suggest that the evaluation of novel chemotherapeutic agents against babesiosis and theileriosis is essential.

Methylene blue (MB), a popular staining dye, has been used against human malaria in the past. MB has also been used to treat several other human diseases, such as Alzheimer's disease and methemoglobinemia (Faley and Chase, 2012; Paban *et al*., 2014). Furthermore, a past study demonstrated that the toxicity of MB was higher for erythroleukemic cells than for normal peripheral blood mononuclear cells, suggesting that this drug has the potential for a safer application in human medicine (Kirszberg *et al*., 2005). One of the oldest components successfully used to treat *B. bigemina* is trypan blue, which is also a staining dye. However, trypan blue has not been used against other bovine parasites (Schoeman, 2009). In the past, several compounds that suppressed the growth of *Plasmodium* parasites were successfully evaluated against *Babesia* and *Theileria* parasites. Although a number of studies detailed the inhibitory effects of MB on *P. falciparum*, the effect of this drug on *Babesia* and *Theileria* parasites remained to be evaluated (Bork *et al*., 2003; Goo *et al*., 2010; Aboulaila *et al*., 2010, 2012; Salama *et al*., 2013). Therefore, in the present study, we conducted an *in vitro* evaluation of MB against *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi*. In addition, an *in vivo* evaluation of MB was conducted against *B. microti* in mouse model.

MATERIALS AND METHODS

Parasites and drugs

B. bovis (Texas strain), *B. bigemina* (Argentine strain), *B. caballi*, *T. equi* (both USDA strains), and *B. microti* (Munich strain) were used in this study (Bork *et al*., 2004). MB was purchased from AppliChem GmbH (Darmstadt, Germany). MB was dissolved in water (Millipore) to prepare a 100 mM stock solution and stored at

-30ºC until further use for *in vitro* growth inhibition assay. Ganaseg was purchased from Ciba-Geigy Japan, Ltd. (Tokyo, Japan). Every 2 days, MB and ganaseg were dissolved in water (Millipore) by dose at 50 and 25 mg/kg of body weight, respectively, and then stored at 4ºC until further use for *in vivo* growth inhibition assay.

Parasite cultivation

The *Babesia* and *Theileria* parasites were maintained using a previously described microaerophilic stationary-phase culture system in purified bovine or equine red blood cells (RBCs) (Bork *et al*., 2004). For the cultivation, medium M199 was used for *B. bovis*, *B. bigemina*, and *T. equi*, while medium RPMI 1640 was used for *B. caballi*. Media were supplemented with 40% bovine (for *B. bovis* and *B. bigemina*) or equine (for *B. caballi* and *T. equi*) sera, 60 U/ml of penicillin G, 60 µg/ml of streptomycin, 0.15 µg/ml of amphotericin B; an exclusively cultured media of *T. equi* was also added to 13.6µg/ml of hypoxanthine (all of the media and supplements were purchased from Sigma-Aldrich, Tokyo, Japan) before cultivation of the parasites. The parasites were maintained under an appropriate environment, as previously described (Avarzed *et al*., 1997; Bork *et al*., 2004).

***In vitro* growth inhibition assay**

The *in vitro* growth inhibitory assays for *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* were conducted as previously described (Salam *et al*., 2013). Briefly, 20 µl of bovine or equine RBCs with 1% parasitemia was added to the 200 µl culture media, which were supplemented with different concentrations (0.01, 0.1, 1,10, 50, and 100 µM) of MB in 96-well culture plates (Nunc, Roskilde, Denmark) in triplicate and incubated for 4 days. A media without MB were used as controls. Every 24 hours, thin blood smears were prepared from each well, and the medium was replaced with a fresh medium containing MB with indicated concentrations. Blood smears were stained with Giemsa and observed under a microscope. The parasitemia was determined by counting infected RBCs among 2000 total RBCs. The half maximal inhibitory concentrations (IC_{50}) of MB for each parasite species were determined by comparing the parasitemia of controls and treated parasites when the control groups had the maximum parasitemia. The experiments were repeated 3 times for each parasite.

Viability test

After 4 days of treatment, 6 µl of RBCs from each well was transferred to new wells containing 14 µl of uninfected bovine or equine RBCs and 200 µl of respective drug-free media and incubated appropriately. The media were replaced every day for another 4 days. Thin blood smears were prepared every day and observed under a microscope for the presence of parasites (Bork *et al*., 2004; Aboulaila *et al*., 2010).

In vivo growth inhibition assay

B. microti was injected into BALB/c mice (Clea, Tokyo, Japan) as previously described (Bork *et al*., 2004). Fifteen mice were divided into 3 groups of 5 mice each and intraperitoneally injected with $1x10^7$ *B. microti*–infected RBCs. Subsequently, 50 mg/kg of MB in 0.2 ml water was orally administered to one group, while 0.2 ml water was orally administered to another group as an untreated control. Finally, 25 mg/kg of ganaseg in 0.2 ml water was subcutaneously administered to the remaining group as a control treatment. All administration was conducted for 6 consecutive days, days 3–8 post-infection, when approximately 1% parasitemia is indicated in the blood of the mice. Parasitemia was monitored in all mice on days 1, 2, 3, 5, 7, 8, 9, 10, 11, 12, 14, 18, 22, 26, and 30 by the Giemsa stain method. The present study was carried out in accordance with the ethics standard relating to the care and management of experimental animals set by the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan.

Statistical analysis

The differences in percentage of parasitemia among different groups were statistically analyzed by Student's *t*-test. A *P* value of <0.05 was considered statistically significant.

RESULTS

In vitro growth inhibitory assay

MB was tested on 4 parasite species of *Babesia* and *Theileria* in the present study. The *in vitro* growths of *B. bovis*, *B. caballi*, and *T. equi* were significantly inhibited ($P<0.05$) by 0.1 µM MB, while *B. bigemina* was significantly inhibited by 0.01 µM MB on day 3 of cultivation. Further, *B. bovis* and *B. bigemina* were completely suppressed by 50 µM MB (Fig. 1A and B), and *B. caballi* and *T. equi* were completely suppressed by 10 µM MB (Fig. 1C and D). The half maximal inhibitory concentration (IC_{50}) of MB against *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* was observed at 0.83±0.02, 0.68±0.09, 0.54±0.14, and 0.49±0.06 µM, respectively (Table 1). In addition, parasites showed severe morphological changes in media with 10 µM concentration of MB on day 2, such as a higher number of loss of typical shapes of parasites and degenerative form to compare with the morphological form of parasites in fresh media on day 2 (Fig. 2 and 3).

Viability test

Drug-free media were used for the viability testing of treated parasites. The findings showed that there were no growths of *B. bovis* or *B. bigemina* that had been previously treated with 10 µM MB. Similarly, *B. caballi* and *T. equi* that had been previously treated with 1 µM MB failed to grow in the viability tests. In contrast, the parasites that were not treated (control) in the growth inhibitory assays grew normally.

Table 1. IC_{50} values of MB

Organisms and mammalian cells	IC_{50} (μM)
B. bovis	0.83±0.02
B. bigemina	0.68±0.09
B. caballi	0.54±0.14
T. equi	0.49±0.06
P. falciparum	0.004[a]
Human monocytic leukemia–derived cell line J-111	26.7[b]

[a] – Pascual et al., 2011
[b] –Atamna et al., 1996

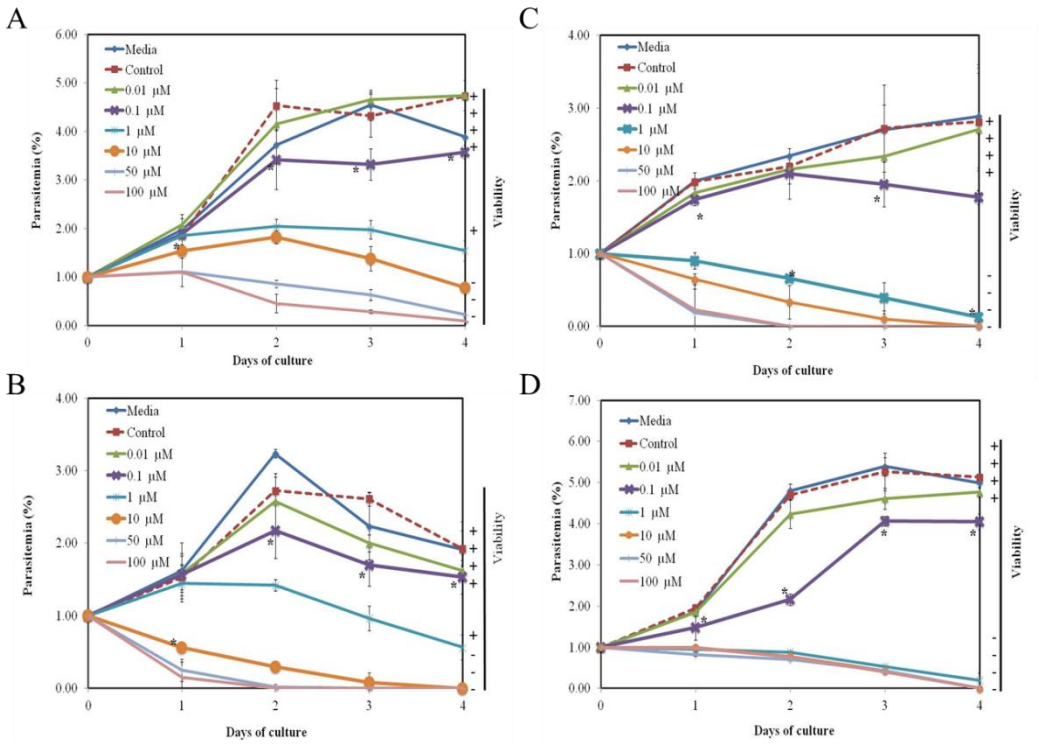

Fig. 1. *In vitro* inhibitory effect of MB on *B. bovis* (A), *B. bigemina* (B), *B. caballi* (C), and *T. equi* (D). Each value represents the mean parasitemia of 3 separate experiments ± standard deviation. Asterisks denote statistically significant differences (P<0.05) between the treated and control cultures.

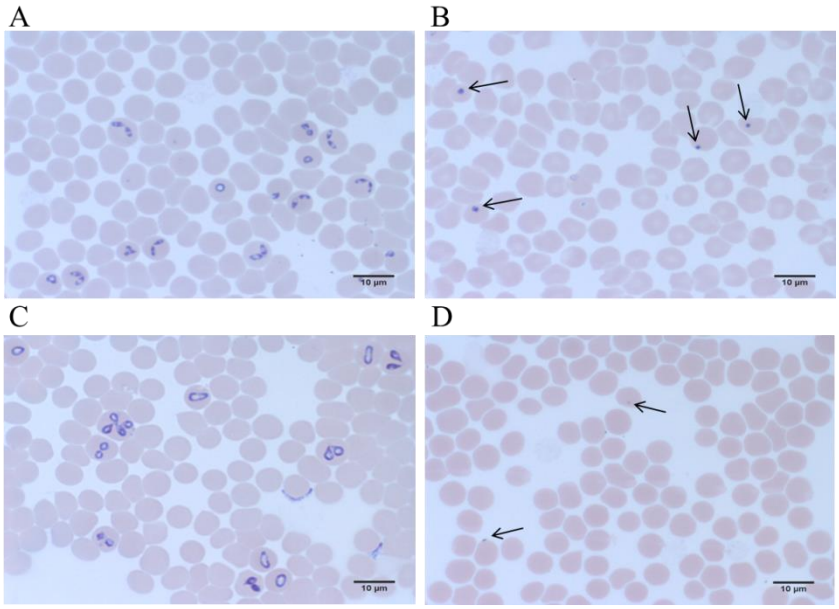

Fig. 2. Light micrographs of MB-treated parasites. Giemsa-stained thin blood smears prepared on the 2nd day of experiments from *in vitro* cultures of *B. bovis* (A and B) and *B. bigemina* (C and D) that were treated with 10 µM MB (B and D) or untreated (A and C). Arrows indicate severe morphological changes in the treated parasites.

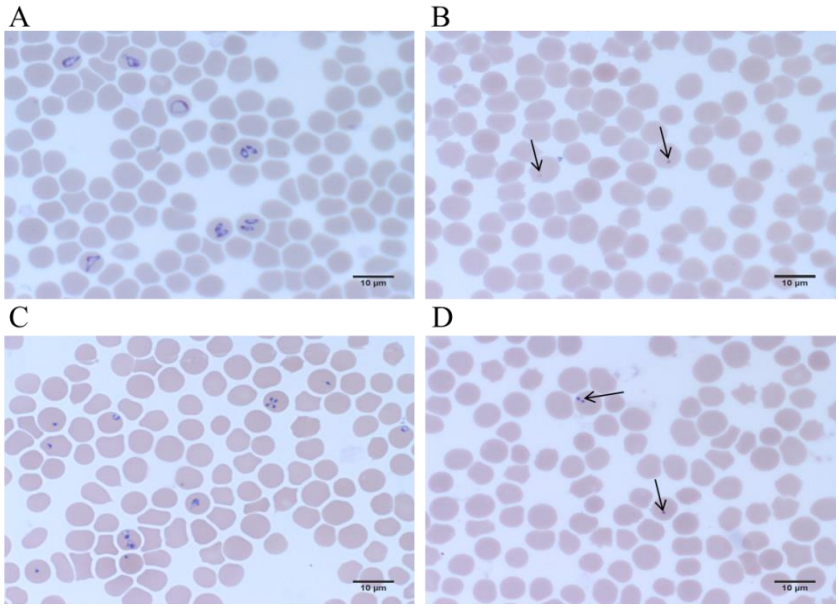

Fig. 3. Light micrographs of MB-treated parasites. Giemsa-stained thin blood smears prepared on the 2nd day of experiments from *in vitro* cultures of *B. caballi* (A and B) and *T. equi* (C and D) that were treated with 10 µM MB (B and D) or untreated (A and C). Arrows indicate severe morphological changes in the treated parasites.

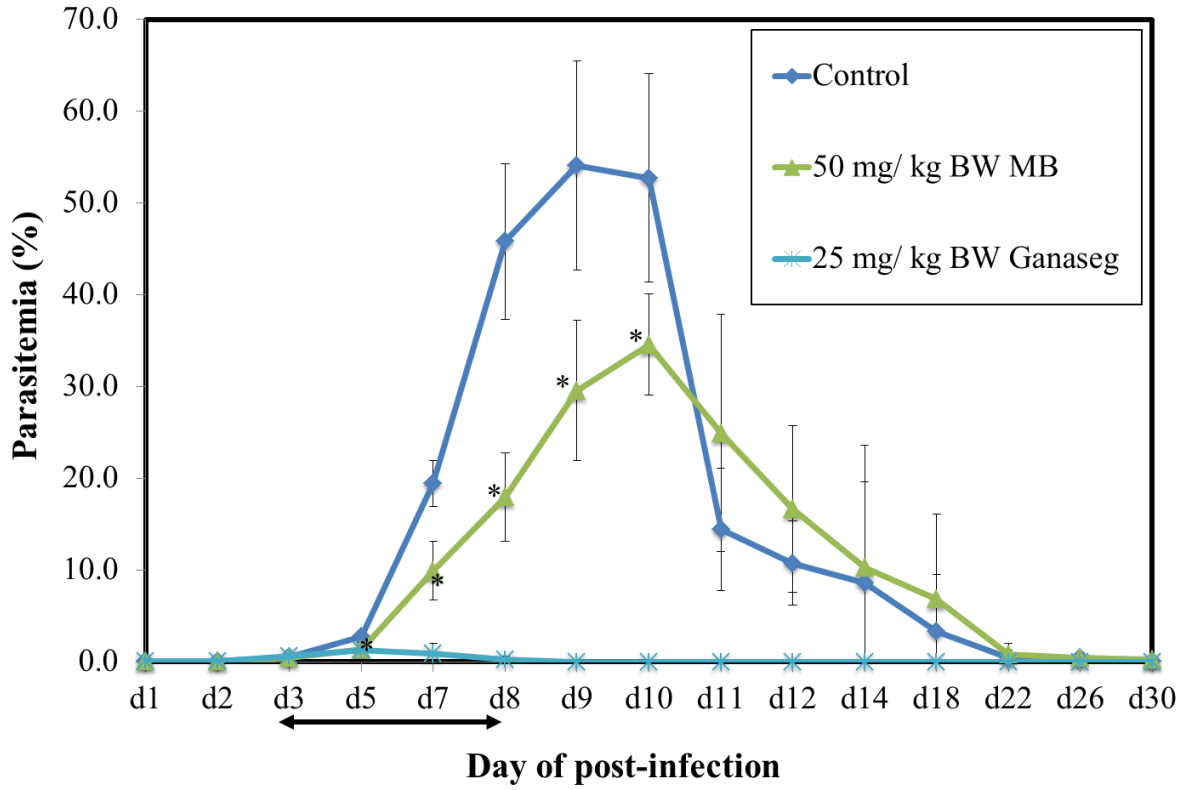

Fig. 4. Groups treated with 50 mg/kg MB or 25 mg/kg ganaseg and untreated groups were observed for parasitemia, with 5 mice in each group. Each value is indicated with the mean ± standard deviation for each group. The statistically significant differences (P<0.05) between the 50 mg/kg MB–treated group and the control group are indicated with asterisks. The arrow is indicated with period of treatment.

In vivo growth inhibition assay

The MB showed high inhibition efficiency on the *in vitro* growth of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* parasites. Therefore, it was evaluated for the *in vivo* inhibition effect of MB against *B. microti* in mice. An *in vivo* growth inhibition assay showed that lower parasitemia was observed with 50 mg/kg MB treatment than in the control group on days 7–10 (Fig. 4). Furthermore, the 50 mg/kg MB–treated group indicated peak parasitemia of 34.6% on day 10, whereas the control group indicated peak parasitemia of 54.1% on day 9. On the other hand, the growth of *B. microti* was inhibited by 36% in mice with 50 mg/kg MB treatment, a delayed peak of parasitemia (day +1) as compared to the control. The growth of *B. microti* was significantly (*t*-test, P<0.05) inhibited on days 5–10 by 50 mg/kg MB treatment as compared to the control group.

DISCUSSION

MB is known to have a many uses in human medicine as a therapeutic agent. Studies related to malaria parasites indicated that MB can inhibit the growth of these parasites. The mechanisms of its antimalarial effects are thought to be due to the selective inhibition of glutathione reductase (Farber *et al*., 1998). As for *Babesia* parasites, the previous study failed to find the encoding region of glutathione reductase; nevertheless, the *B. bovis* are also predicted to possess similar enzymatic activity as those of the known thioredoxin reductase (Regner *et al*., 2014). The *P. falciparum* glutathione reductase gene sequence shared 40.2 and 44.5% identity, respectively, with thioredoxin and glutathione reductase family protein gene sequences of *B. bovis* (GenBank accession number: XM_001608819) and *T. equi* (GenBank accession number: XM_004831637). This observation suggested that MB might have a similar inhibitory effect on *Babesia* and *Theileria* parasites. The findings of the present study confirmed this hypothesis, as all of the parasites tested were suppressed by MB.

The IC_{50} values determined in the present study were higher than those estimated for *Plasmodium* parasites. For example, the IC_{50} value of MB for *T. equi* (0.49±0.06 μM), the lowest value among parasites that were tested in this study, was nearly 100 times higher than that of *P. falciparum* (0.004 μM) (Pascual *et al*., 2011). This observation suggests that *P. falciparum* is relatively more susceptible to MB than *Babesia* and *Theileria* parasites. However, the IC_{50} values obtained in the present study are well below the IC_{50} value for mammalian cells (Table 1) (Atamna *et al*., 1996).

A higher dose of 50 mg/kg MB treatment showed lower efficiency (36% inhibition) against *B. microti* as compared to the 25 mg/kg ganaseg treatment (97.8% inhibition) in this study (Fig. 4). Additionally, the mouse urine was stained to become bluish in coloration under 50 mg/kg MB treatment group during day 4–12 post-infection (days 2–10 post-primary treatment) (Fig. S1). The coloration problem associated with the use of MB is its green or blue color. People treated with MB produced bluish urine (Meissner *et al*., 2005). On the other hand, the urine color became normal on day 13 after the last treatment (day 5 post-treatment). Possible discoloration of livestock products from animals treated with MB can probably be ruled out.

In summary, the present study examined the effects of MB on *Babesia* and *Theileria* parasites. The growth of the parasites was inhibited, with the IC_{50} values well above those for mammalian cells. Nevertheless, methylene blue might not be used for against *Babesia* and *Theileria* parasites because the *in vivo* growth of *B. microti* showed that a higher dose of MB had low efficiency.

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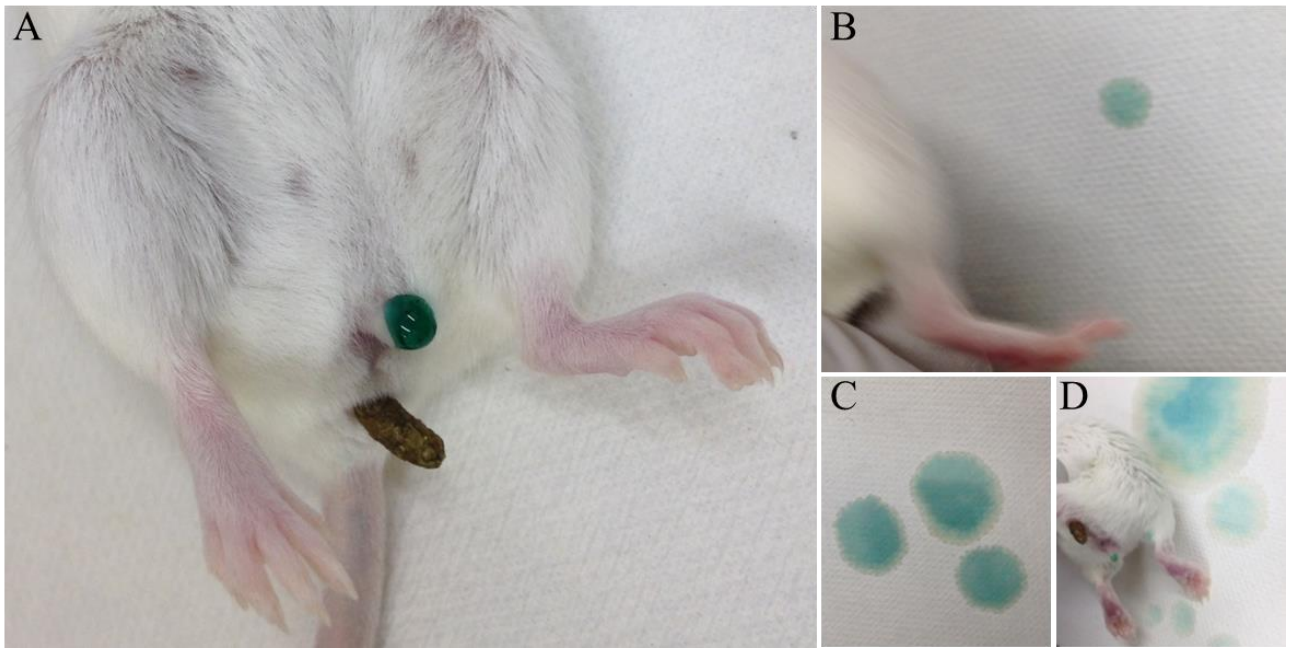

Fig. S1. Fifty mg/kg MB–treated mouse urine was stained with blue coloration on day 8 post-infection (day 6 after last treatment) (A). The urine was dropped onto paper wipes for appropriate recognition (B, C, and D).