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## Research Article

# Evaluation of The *In Vitro* And *In Vivo* Inhibitory Effects of Enrofloxacin On the Growth of *Babesia* Species and *Theileria Equi*

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### ABSTRACT

**Objectives:** Enrofloxacin, a fluoroquinolone antibiotic, is an inhibitor of prokaryotic topoisomerase II with antibacterial and antiparasitic activities. The study aimed to evaluate the inhibitory effect of enrofloxacin on *Babesia* species and *Theileria equi* *in vitro* and *in vivo*.

**Methods:** The inhibitory effects of enrofloxacin were evaluated *in vitro* cultures using *in vitro* inhibition assay of three *Babesia* species and *Theileria equi*; furthermore, the *in vivo* inhibitory effect of enrofloxacin was evaluated in the mice model of *Babesia microti*.

**Results:** The IC<sub>50</sub> values of enrofloxacin were 4.9, 4.5, 4, and 3.9 nM for *B. bovis*, *B. bigemina*, *B. caballi*, and *B. equi*, respectively. Enrofloxacin at a dose rate of 10 mg/kg resulted in a 92.9 % inhibition of *Babesia microti* growth in BALB/c mice. Combination therapy of enrofloxacin at a dose rate of 5 mg/kg with diminazene aceturate at a dose rate of 12.5 mg/kg resulted in 93.83 % inhibition of *Babesia microti* growth in BALB/c mice.

**Conclusions:** Enrofloxacin might be used for drug therapy in babesiosis.

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### Introduction

Piroplasmiasis, tick-transmitted disease, affects bovine and equine hosts. *Theileria equi*, *Babesia caballi*, *Babesia bigemina*, and *Babesia bovis* are the major causes of piroplasmiasis in equine and bovine hosts all over the world. The infection leads to seriously profitable losses to the animal industry worldwide. The clinical disease manifested with malaise, fever, hemolytic anemia, jaundice, enlarged lymph nodes, and hemoglobinuria [1]. *Babesia microti*, a rodent *Babesia*, infects humans in the United States of America and Europe [2]. The infection is controlled by the diagnosis and drug treatment. The currently used chemotherapeutic drugs such as diminazene aceturate, quinuronium sulfate, and imidocarb

dipropionate have drawbacks namely toxicity of the host [3]. Therefore, the advance of recent effective drug therapies against piroplasmiasis and devoid of toxicity to the hosts is highly desired.

The apicomplast originates from cyanobacteria. The biosynthetic pathways in it are different from the similar eukaryotic paths in the mammals. Therefore, it considers as a good drug target [4]. The apicomplast keeps the bacterial housekeeping machinery in *Babesia*, *Theileria*, *Plasmodium falciparum*, and *Toxoplasma gondii*, including DNA replication, transcription, and translation pathways [5-8]. This machinery provides a good drug target.

Enrofloxacin inhibits DNA gyrase, a prokaryotic type II topoisomerase,

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therefore, blocks prokaryotic DNA replication by hindering the untangling DNA during replication, and results in the linearization of the circular DNA, thus causing the death of prokaryotic organisms without affecting the mammalian topoisomerase [9-11]. DNA gyrase is made of two subunits, namely, DNA gyrase subunit A and DNA gyrase subunit B. Enrofloxacin has antibacterial, anti-anaplasma, antileishmanial, antitrypanosomal, anti-neospora, and anti-toxoplasma activities [12-18]. It is used clinically for treating bacterial infections in farm animals; therefore, it will be of economic value for treating piroplasmosis caused by *Babesia* and *Theileria* species by clinically available medicine. The present study aimed to value the suppressive results of enrofloxacin on the *in vitro* growth of three *Babesia* species and *T. equi* and on the *in vivo* growth of *B. microti*.

## Materials and methods

### I Chemical reagents

A solution of 100 mM enrofloxacin (Sigma-Aldrich, USA) in dimethyl sulfoxide (DMSO) was prepared and stored at  $-30^{\circ}\text{C}$ . For *in vivo* studies, enrofloxacin 2.5 % injectable solution (Baytril<sup>®</sup>) was picking up from Bayer AG (Leverkusen, Germany). An operational store solution of 10 mM Diminazene aceturate (Ganaseg) (Ciba-Geigy Japan Ltd., Tokyo, Japan) dissolved in DDW was prepared and stored at  $-30^{\circ}\text{C}$  until required for use.

### II Rodent *Babesia* and mice

*B. microti*, Munich strain, was conserved by serialized passage in mice [19]. Thirty (8 weeks old) BALB/c female mice were procured (CLEA Japan) and used for the *in vivo* experiments.

### III *In vitro* cultivation of *Babesia* parasites

Enrofloxacin was anticipated for its chemotherapeutic influence against *B. bovis* (Texas strain), *B. bigemina* (Argentina strain), *B. caballi* and *T. equi* (U.S. Department of Agriculture) [20-23]. Parasites were cultivated in the horse or the cow red blood corpuscles by means of a continuous micro-aerophilous stationary phase culture system [19]. The M199 medium (Sigma-Aldrich, Japan) was employed for *B. bovis*, *B. bigemina*, and *T. equi*. It was accompanied with bovine or equine serum at 40 %, penicillin G at 60 U/ml, streptomycin at 60  $\mu\text{g/ml}$ , and amphotericin B at 0.15  $\mu\text{g/ml}$  (Sigma-Aldrich). As an essential complement, hypoxanthine (ICN Biomedicals, Inc., Aurora, OH) was supplementary, at 13.6 mg/ml, to the *T. equi* culture. The culture of *B. caballi* consisted of the medium RPMI 1640 enhanced with 40 % horse serum, antibiotics, and amphotericin B [24].

### IV *In vitro* growth inhibition assay

The *in vitro* growth inhibition assay was completed as earlier stated [25]. Cultures of *B. bigemina*, *B. caballi*, *B. bovis*, and *T. equi*, with about 5 % parasitemia, were diluted with proper uninfected erythrocytes to an initial parasitemia of 1%. The growth inhibition assay was accomplished in 96-well plates comprising 20  $\mu\text{l}$  of packed erythrocytes inoculum and 200  $\mu\text{L}$  of a fitting culture medium containing 0.005, 0.05, 0.1, 1, 5, 50, 100  $\mu\text{M}$  for *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* of enrofloxacin.

The concentrations used were based on a primary experiment. For positive control, diminazene aceturate was used at 5, 10, 50, 100, 1000, 1500 or 2000 nM [19]. For negative experimental control, cultures lack the drug and cultures containing only double-distilled water (0.01 %, for enrofloxacin and 0.02 %, for diminazene aceturate) were ready. The experiments were conveyed thrice in triplicate. For four days, cultures were kept at  $37^{\circ}\text{C}$  in an incubator with air formula of 5 %  $\text{CO}_2$ , 5 %  $\text{O}_2$ , and 90 %  $\text{N}_2$ . Every day, 200  $\mu\text{l}$  of fresh medium, containing the fitting drug concentration, were used to replace the old culture medium. Parasitemia was calculated by examining 1,000 erythrocytes. Morphological changes were detected in the treated *Babesia* parasites comparable with the control microscopically. On the third day of *in vitro* culture, the 50 % inhibitory concentration ( $\text{IC}_{50}$ ) values were estimated by the curve-fitting technique [24].

### V Viability test

Next, to the fourth day of treatment, 6  $\mu\text{L}$  of new cow or horse erythrocytes was added to 14  $\mu\text{L}$  of erythrocytes from the earlier drug-treated cultures in 200  $\mu\text{l}$  of a renewed growth medium without the medication. The replacement growth medium was changed on a daily basis for the ensuing 10 days, and parasite reactivation was microscopically examined daily after exclusion of the drugs [22].

### VI Effect of enrofloxacin on host erythrocytes

The toxic effect of enrofloxacin was appraised on host erythrocytes as before defined [22]. 100  $\mu\text{M}$  concentration of enrofloxacin was incubated with bovine and equine red blood cells for 3 hours at  $37^{\circ}\text{C}$ ; at that time RBCs were washed three times with media alone and used for the culture of *Babesia* parasites for 3 days. The control untreated cells controlled in the same style as the pretreated cells. The form of parasite growth in pretreated erythrocytes was detected and likened with control untreated cells.

### VII *In vivo* growth inhibition assay

The enrofloxacin *in vivo* inhibition assay for *B. microti* in BALB/c mice was performed twice following a method previously described with some modifications. Twenty female BALB/c mice of 8 weeks old were alienated into four groups, each containing five mice, and intraperitoneally injected with  $1 \times 10^7$  *B. microti*-infected RBCs [14, 22]. After the inoculated mice presented almost 1 % parasitemia, mice in the investigated groups were received regular doses for five days.

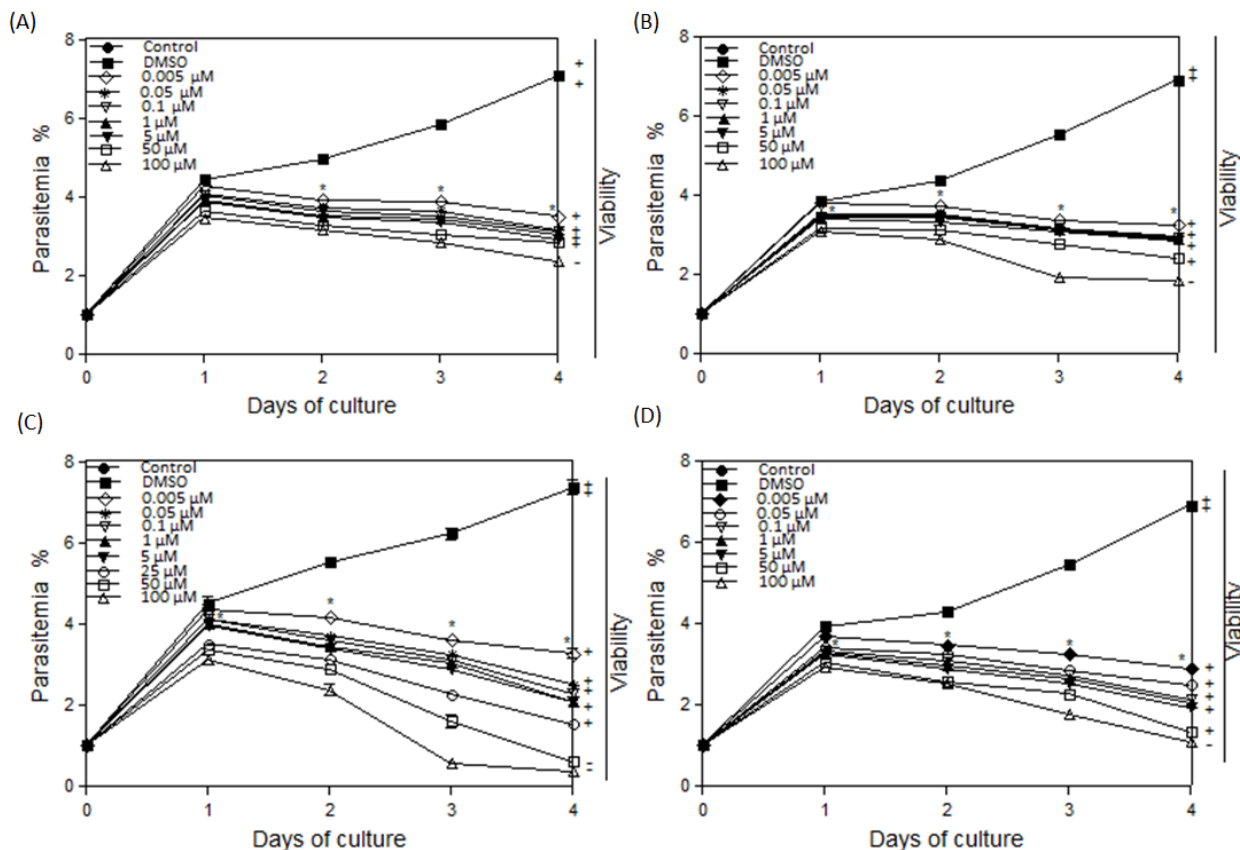
Diminazene aceturate was melted in DDW (12.5 %), then diluted in DDW prior to inoculation while enrofloxacin was dissolved in phosphate buffered saline (PBS) (1.33 % and 2.66 % v/v) directly prior to inoculation. In the negative control, PBS was administered. In the first and second groups, enrofloxacin was I.P. injected at dosages of 10 mg/kg and 5 mg/kg body weight, respectively, in 0.3 ml of PBS [22]. A 0.3 ml PBS was injected intraperitoneally to the control group. Diminazene aceturate (Ganaseg, Ciba-Geigy, Japan Ltd.) at a dose of 12.5 mg/kg and 25 mg/kg was S/C injected to the second and third experimental groups, respectively in 0.1 ml DDW.

The parasitemia levels in all mice were examined every day till the day

22 P.I by inspection of 1,000 erythrocytes in blood smears obtained from the tail vein. The animal experiments were led in accord with the Standard Pertaining 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.

VIII Statistical analysis

Variations in parasitemia percentages in the *in vitro* and the *in vivo* experiments were analyzed using the independent Student's t-test with JMP statistical software (SAS Institute, Inc., USA). A P value of < 0.05 was counted statistically significant.



**Figure 1:** Inhibitory effects of different concentrations of enrofloxacin on the *in vitro* growth. (A) *B. bovis*, (B) *B. bigemina*, (C) *B. caballi*, and (D) *T. equi*. Each value represents the mean ± standard deviation in triplicate. These curves represent the results of three experiments carried out in triplicate. Asterisks indicate a significant difference (Student's t-test; \* P < 0.05) between enrofloxacin-treated and control cultures. Regrowth after 10 days was indicated as viable (+) and dead (-).

**Table 1.** IC<sub>50</sub> values of enrofloxacin and diminazene aceturate for *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi*

	IC <sub>50</sub> (nM) <sup>a</sup>	
	Enrofloxacin	Diminazene
<i>B. bovis</i>	5 ± 0.2	300 ± 30
<i>B. bigemina</i>	4.9 ± 0.1	190 ± 20
<i>B. caballi</i>	4 ± 0.2	10 ± 2
<i>T. equi</i>	3.9 ± 0.1	710 ± 15
HFF cells	265130 <sup>*</sup>	ND
Mouse macrophage	141280 <sup>†</sup>	

<sup>a</sup> IC<sub>50</sub> values expressed as drug concentration are in nanomolar of the growth medium and were determined on day 4 of *in vitro* culture using a curve fitting technique. IC<sub>50</sub> values represent the mean and standard deviation of 3 separate experiments

<sup>\*</sup> non-toxic concentration Barbosa et al [18]

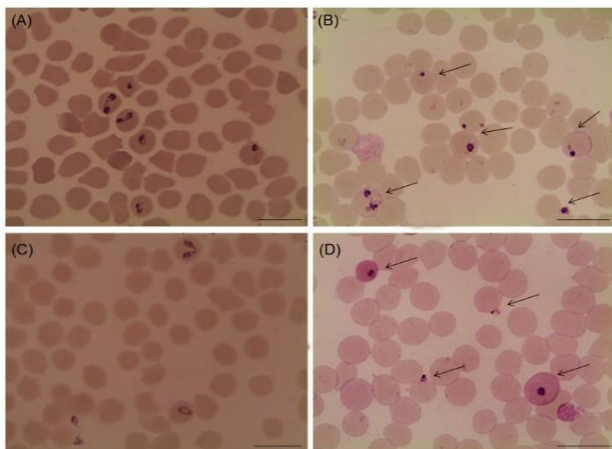
<sup>†</sup> Moderately toxic concentration Bianciardi et al [14]

ND not determined

## Results

### I *In vitro* growth inhibition assay

Parasites growth was significantly inhibited by enrofloxacin at 5 nM for *B. bovis* (Fig. 1A), *B. bigemina* (Fig. 1B), *B. caballi* (Fig. 1C), and *T. equi* (Fig. 1D) at days 2-4 of treatment. Similarly, diminazene aceturate treatment with 5 nM significantly ( $P < 0.05$ ) subdued the *in vitro* progress of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi*. The viability of parasites after drug removal for 10 days showed no increase of parasitemia at concentrations of 100  $\mu\text{M}$  for *T. equi* and bovine *Babesia* and 50  $\mu\text{M}$  for *B. caballi* (Fig. 1). Parasites exposed to lower drug concentrations regrow when the drug was removed. Growth was not restarted by diminazene aceturate at concentrations of 50 nM (*B. caballi*) and 1500 nM (*T. equi* and bovine *Babesia*) (information not displayed). Enrofloxacin and diminazene  $\text{IC}_{50}$  values for different *Babesia* species are shown (Table 1). Parasites exposed to only DMSO or DDW in the cultures had similar growth pattern to the control.



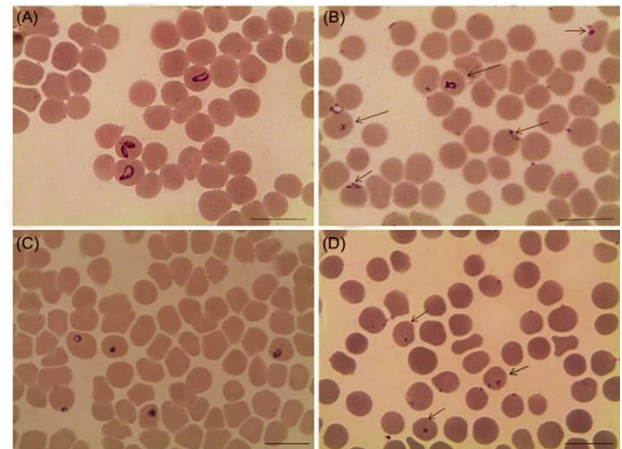
**Figure 2:** Light micrographs of *Babesia bovis* and *Babesia bigemina* treated with 50  $\mu\text{M}$  enrofloxacin in *in vitro* cultures. (A) *Babesia bovis* control, (B) enrofloxacin-treated cultures, (C) *Babesia bigemina* control, and (D) enrofloxacin-treated cultures. The drug-treated cultures showed higher numbers of degenerated parasites indicated by arrows than the control cultures. Micrographs were taken on day4 of treatment. Bars, 10  $\mu\text{m}$ .

The differences in the parasite morphology due to treatment were compared. In enrofloxacin-treated *B. bovis* cultures, the parasites appeared dot-shaped (Fig. 2B) relative to normal parasites in the DMSO-treated parasites (Fig. 2A). A similar effect was observed in enrofloxacin-treated *B. bigemina* (Fig. 2D), *B. caballi* (Fig. 3B), and *T. equi* (Fig. 3D) cultures (not shown). Bovine and equine erythrocytes were not distorted by the treatment with the highest concentration of enrofloxacin (100  $\mu\text{M}$ ). The progression of the parasites was similar in untreated erythrocytes and the treated ones (data not shown).

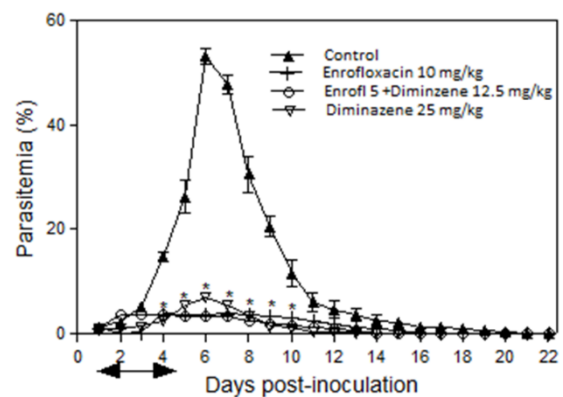
### II *In vivo* effect of enrofloxacin on *B. microti* infection

Enrofloxacin was evaluated for *in vivo* efficacy for *B. microti* in mice. The enrofloxacin significantly decreased the parasitemia in treated groups than the negative control group ( $P < 0.05$ ) from days 4 to 10 p.i. (Fig. 4). Parasitemia reach the peak of 7 % at 25 mg/kg diminazene

aceturate at 6 days p.i. and 3.74 % at 10 mg/kg enrofloxacin and 3.28 % at 5 mg/kg enrofloxacin plus 12.5 mg/kg diminazene aceturate at 7 days p.i., in contrast to 53.12 % in the negative control group (DMSO) at 6 days p.i. (Fig. 4).



**Figure 3:** Light micrographs of *Babesia caballi* and *Theileria equi* treated with 50  $\mu\text{M}$  enrofloxacin in *in vitro* cultures. (A) *Babesia caballi* control, (B) enrofloxacin-treated cultures, (C) *Theileria equi* control, and (D) enrofloxacin-treated cultures. The drug-treated cultures showed higher numbers of degenerated parasites indicated by arrows than the control cultures. Micrographs were taken on day4 of treatment. Bars, 10  $\mu\text{m}$ .



**Figure 4:** Inhibitory effects of I.P. enrofloxacin 14.5 mg/kg and S.C. diminazene aceturate 25 mg/kg on the *in vivo* growth of *Babesia microti* for observations of five mice per experimental group. Each value represents the mean  $\pm$  S.D for two experiments. Asterisks indicate a significant difference (Student's t-test; \*  $P < 0.01$ ) from days 4 to 8 post-inoculation between enrofloxacin-treated and dimethyl sulfoxide (DMSO) control group.

## Discussion

In the present study, enrofloxacin depressed the *in vitro* progression of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi*. The existence of greater concentrations of enrofloxacin in the culture entirely repressed the expansion of *T. equi*, *B. caballi*, *B. bovis*, and *B. bigemina*. The inhibitory effect was due to enrofloxacin as the DMSO had no influence on the parasites' growth. *Babesia* and *T. equi* had similar sensitivity to

enrofloxacin.

The IC<sub>50</sub>s of enrofloxacin for *T. equi* and *Babesia* species were lower than those of diminazene reported in this study. The IC<sub>50</sub>s of enrofloxacin for *T. equi* and *Babesia* species were lesser than other formerly experienced antibabesial drugs and lower than its IC<sub>50</sub> for *B. divergens*. The IC<sub>50</sub> values of enrofloxacin for *T. equi* and *Babesia* species were lesser than the IC<sub>50</sub> values of other babesicidal drugs: quinuronium sulfate and imidocarb dipropionate [19, 21, 22, 24-36]. The IC<sub>50</sub> values of enrofloxacin for *T. equi* and *Babesia* species ( $\leq 5$ nM) are very low compared with a concentration of 265.13  $\mu$ M (200  $\mu$ g/ml) that did not affect the viability of HFF cells [18]. Furthermore, the IC<sub>50</sub> values of enrofloxacin for *T. equi* and *Babesia* species were very low compared with a moderately toxic concentration of 141.28  $\mu$ M (50  $\mu$ g/ml) for mouse macrophage (Bianciardi et al., 2004) which is > 25000 times the IC<sub>50</sub> values reported in this study. Therefore, enrofloxacin will be safe for treating piroplasmosis in animals and human.

Enrofloxacin showed good *in vitro* inhibitory effects on *T. equi* and three *Babesia* species and *in vivo* inhibitory effects on *Leishmania infantum* and *Trypanosoma congolense*; thus, we evaluated the *in vivo* repressive activity of enrofloxacin on *B. microti* in mice. Enrofloxacin inhibited the growth of *B. microti* [14, 16]. Treatment with 10 mg/kg B.W. resulted in 92.9% inhibition of *B. microti* growth which is agreement with previous studies where enrofloxacin-treated the infection with *T. gondii* in *Calomys callosus* at an amount of 3 mg/kg B.W. and treated *L. infantum* in dogs at a dosage of 20 mg/kg B.W. indicating that enrofloxacin may be practical for babesiosis [18, 14]. Enrofloxacin was safe to the treated mice that did not show signs of toxicity such as ruffled fur or weight loss and were alive after the experiment. These findings were similarly stated in a preceding study where enrofloxacin at 20 mg/kg B.W. orally for 30 days treated dogs inoculated by *L. infantum* without toxic side effects [14]. Combined treatment of enrofloxacin at 5 mg/kg with diminazene aceturate at 12.5 efficiently inhibited the progression of *B. microti* and this in accord with results of previous studies that used a mixture of enrofloxacin with other drugs such as doxycycline, metronidazole, and isometamidium chloride to control several protozoan infections [13, 14, 16, 37]. Therefore, combination therapy of enrofloxacin might be used for babesiosis. In conclusion, enrofloxacin inhibited the *in vitro* multiplication of *T. equi* and *Babesia* species and the *in vivo* growth of *B. microti* in BALB/c mice. Enrofloxacin may be employed alone or in combination as a chemotherapeutic drug for babesiosis and theileriosis.

### Conflicts of interest

No conflict of interest

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