ORIGINAL RESEARCH

Inhibitory effects of fluoroquinolone antibiotics on *Babesia divergens* and *Babesia microti*, blood parasites of veterinary and zoonotic importance

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Aim: This study aimed to evaluate the inhibitory effects of fluoroquinolone antibiotics, including enrofloxacin, enoxacin, trovafloxacin, norfloxacin, and ofloxacin, on the in vitro and in vivo growth of *Babesia divergens* and *Babesia microti* parasites, respectively.

Materials and methods: The in vitro and in vivo inhibitory effects of fluoroquinolone antibiotics against *B. divergens* and *B. microti*, respectively were evaluated using fluorescence-based assay. Additionally, combination therapies of highly effective fluoroquinolone antibiotics (enrofloxacin, enoxacin, and trovafloxacin) with diminazene aceturate, luteolin, or pyronaridine tetraphosphate were tested on the in vitro cultures of *B. divergens*.

Results: Enrofloxacin, trovafloxacin, and enoxacin were the most effective fluoroquinolones against the in vitro growth of *B. divergens*, followed by norfloxacin and ofloxacin. Furthermore, a combination of enoxacin or trovafloxacin with either diminazene aceturate, luteolin, or pyronaridine tetraphosphate significantly enhanced the inhibitory effect on the growth of *B. divergens* in in vitro cultures. In mice infected by *B. microti*, enoxacin and diminazene aceturate combination therapy exhibited a potential antibabesial effect.

Conclusion: These results suggest that safe and cheap fluoroquinolone, such as enoxacin, might be used for the treatment of clinical cases caused by *Babesia* spp. in animals or humans.

Keywords: *Babesia*, fluoroquinolones, in vitro, in vivo

Introduction

Babesia is a protozoan parasite transmitted by ticks; it is a major pathogen that infects the erythrocytes of a wide range of economically valuable animals as well as human erythrocytes.[1] Babesiosis is a severe, rapidly fatal disease typified by fever, hemolytic anemia, and hemoglobinuria that causes severe economic losses in the livestock industry.[1,2] *Babesia microti* and *Babesia divergens* are blood parasites that infect rodents and cattle, respectively, and have zoonotic importance.[1] *B. microti* is the main etiologic agent recognized for babesiosis in humans in the USA and *B. divergens* is a serious cause of babesiosis in Europe.[3]

Recently, antibabesial drugs commonly used in veterinary medicine have shown problems regarding parasite resistance and toxicity to the host.[4] At the same time, many of the most effective drugs against *B. divergens* have been withdrawn from the market due to safety or residue problems.[3] Moreover, therapeutic failures have been reported in some severe cases of human babesiosis after the administration of clindamycin, azithromycin, quinine, and tetracycline.[3] Therefore, there is an urgent need to develop more effective drugs against *Babesia* parasites that have both veterinary and zoonotic importance. Fluoroquinolones are bactericidal drugs that have the ability to interfere with the

replication of an organism's DNA by inhibiting pathogen DNA gyrases and topoisomerases.[5] Following this pattern, two gene-encoding gyrases (A and B) are present within apicomplexan parasites, with the remarkable exception of *Cryptosporidium* sp., and are driven to their essential plastid-like organelles, the apicoplasts.[6] In fact, apicomplexan apicoplast organelles play a critical role in cell survival in the later stages of parasite evolution.[6] Taken together, there is no ortholog to the apicoplast DNA gyrase of apicomplexan parasites in mammals;[6,7] thus, this enzyme provides an attractive target for drug development. Additionally, DNA topoisomerases (Top) are important enzymes required to produce a more relaxed DNA and thus play a critical role in DNA packaging.[8] Therefore, in the present study, fluoroquinolone antibiotics, including enrofloxacin, enoxacin, trovafloxacin, norfloxacin, and ofloxacin, were selected to evaluate their inhibitory effects against the growth of *B. divergens* in vitro and *B. microti* in vivo.

Materials and methods
Ethical approval
All experimental protocols in this study were approved by the Animal Care and Use Committee, Obihiro University of Agriculture and Veterinary Medicine (approval no. 27-65). All experiments were conducted in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Parasites
A German bovine strain of *B. divergens*[9] was used for the in vitro experiment and cultivated in purified bovine red blood cells (RBCs) using a microaerophilic, stationary-phase culture system.[9] The Munich strain of *B. microti*[10] was used for the in vivo experiment.

Chemical reagents
SYBR Green I (SGI) nucleic acid stain (10,000×; Lonza, Rockland, ME, USA) was stored at –20°C and thawed before use. A lysis buffer consisting of Tris (130 mM; pH 7.5), EDTA (10 mM), saponin (0.016%; W/V), and TritonX-100 (1.6%; V/V) was prepared in advance and stored at 4°C. Diminazene aceturate (DA; Novartis International AG, Basel, Switzerland), tetracycline hydrochloride, and chloroquine diphosphate (both drugs from Sigma-Aldrich Co., St Louis, MO, USA) were used as positive control drugs. Enrofloxacin (Figure 1A), enoxacin (Figure 1B), trovafloxacin (Figure 1C), norfloxacin (Figure 1D), ofloxacin (Figure 1E), luteolin, or pyronaridine tetraphosphate

(PYR) (all drugs from Sigma-Aldrich Co.) was prepared as a 100 mM stock solution and stored at –20°C until use.

Evaluation of fluoroquinolone antibiotics against *B. divergens* in vitro
The in vitro Babesia fluorescence assay (BFA) was performed as previously described by Rizk et al.[9] Briefly, Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich Co.) alone or mixed with the indicated concentrations, 0.25, 0.5, 1, 5, and 10 μM for DA or 0.5, 10, 50, 100, and 400 μM for other fluoroquinolone antibiotics (enrofloxacin, enoxacin, trovafloxacin, norfloxacin, and ofloxacin), was used for the cultivation of parasitized RBCs (pRBCs) in double 96-well plates (Nunc, Roskilde, Denmark) at 5% hematocrit (HCT) for 4 days in triplicate wells for each concentration of the drug. On the fourth day of culture, a lysis buffer containing 2×SGI was added to each well of the first 96-well plate with gentle mixing. For a viability test, on the fourth day of treatment, 3.5 μL of the culture of untreated pRBCs, treated pRBCs, and uninfected RBCs on the second plate was removed and replaced with parasite-free bovine RBCs (3.5 μL) and suspended in 100 μL of a fresh growth medium without drugs. Then the plate was incubated at 37°C for the next 4 days. Next, the fluorescence values were determined using a fluorescence plate reader (Fluoroskan Ascent; Thermo Electron Informatics, Philadelphia, PA, USA) at 485 nm and 518 nm excitation and emission wavelengths, respectively. Each experiment was repeated three times.

In vitro drug combination test
Combination therapies of highly effective fluoroquinolone antibiotics (enrofloxacin, enoxacin, and trovafloxacin) with DA, luteolin, or PYR were tested on the in vitro cultures of *B. divergens*. Fluoroquinolone antibiotics/DA, luteolin, or PYR combinations (M1, M2, M3, and M4, respectively) were prepared as previously described[11,12] with some modifications. Combinations were based on the calculated IC_{50} values obtained from the in vitro BFA (Table S1). Three separate trials were performed, consisting of triplicate experiments for each drug combination over a period of 4 days using 5% HCT. On the fourth day of culture, the fluorescence values were calculated after adding a lysis buffer containing 2×SGI to each well on the 96-well plate.

Chemotherapeutic evaluation of fluoroquinolone antibiotics in mice
Fluoroquinolone antibiotics were administered in an in vivo BFA for *B. microti* in BALB/c mice (CLEA, Tokyo, Japan), following a method described by Rizk et al.[10] Thirty-five
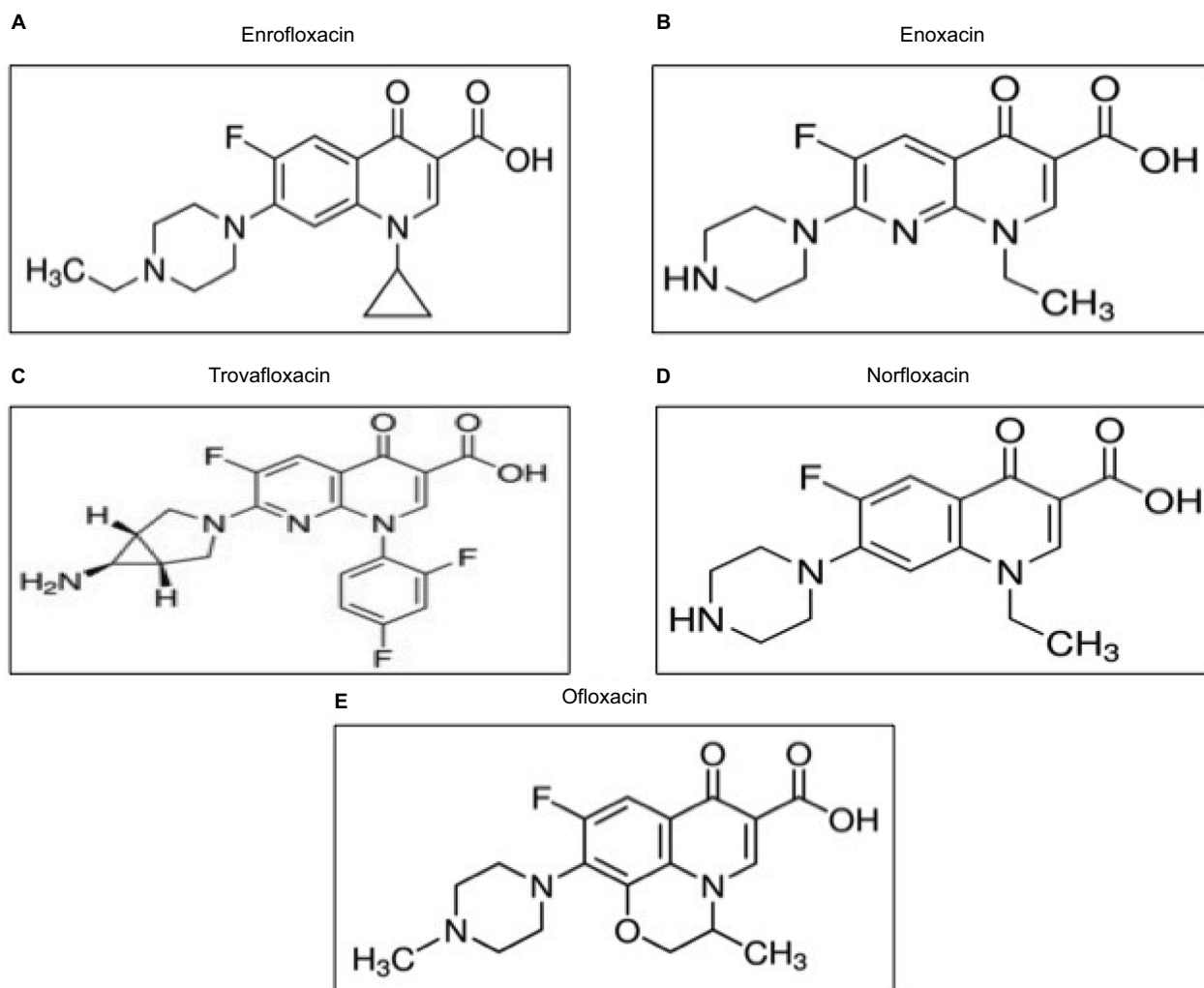
Figure 1 Chemical structures of fluoroquinolone antibiotics used in this study: (**A**) enrofloxacin, (**B**) enoxacin, (**C**) trovafloxacin, (**D**) norfloxacin, and (**E**) ofloxacin.

8-week-old female mice were kept under specific pathogen-free conditions and divided equally into seven groups. Mice in the first six groups were inoculated intraperitoneally with 1×10^7 *B. microti*-infected RBCs, while the mice in the seventh group remained without infection and were used as a blank control. When the infected mice showed ~1% parasitemia, mice in the experimental groups received daily injections of the specific drugs for 5 days. All administered drugs were dissolved in double-distilled water (DDW), and DDW was administered subcutaneously to the mice in the first group as a placebo control.[10] DA was administered to the second group subcutaneously at a dose rate of 25 mg·kg^{-1} as a reference drug control. Mice in the third, fourth, and fifth groups received daily subcutaneous injections of nontoxic doses[15] of enoxacin (100 mg·kg^{-1}), norfloxacin (150 mg·kg^{-1}), and ofloxacin (700 mg·kg^{-1}), respectively. Mice in the sixth group were treated with a combination therapy consisting of 50 mg·kg^{-1} enoxacin and 10 mg·kg^{-1} DA. Both drugs were administered

subcutaneously in the same inoculation period. The emitted fluorescence signals were used as an indicator of the parasitemia percentages in all mice and monitored every 48 hours until 20 days post-inoculation or the cessation of parasitemia.

Role of fluoroquinolone antibiotics in the regression of anemia associated with *Babesia*

HCT values, hemoglobin levels, RBC counts, mean corpuscular volume, mean corpuscular hemoglobin, and red blood cell distribution width were used as indicators of the development of anemia in mice treated with fluoroquinolone antibiotics. Ten microliters of venous tail blood samples was collected from all mice as previously described. In all groups, the hematological parameters were monitored every 96 hours using the Celltac α MEK-6450 automatic hematology analyzer (Nihon Kohden Corporation, Tokyo, Japan).

Polymerase chain reaction (PCR) detection of *B. microti* in mice treated with DA and enoxacin combination therapy

Nested PCR assays targeting the *B. microti small subunit rRNA (ss-rRNA)* gene were used to detect parasite DNA in blood and tissue (spleen, heart, lung, and kidney) samples collected from mice treated with an enoxacin/DA combination, DA alone, and DDW (positive control) on day 20 post-infection (pi). DNA was extracted from the tissues collected from treated mice using a NucleoSpin tissue kit (Macherey-Nagel, Düren, Germany). DNA extracted from the blood of *B. microti*-infected mice treated with DDW (positive control) using a QIAamp DNA Blood Mini Kit (Qiagen, Tokyo, Japan) served as a positive control. PCR cycling was performed as previously described by Rizk et al.[10]

Statistical analysis

The differences in the fluorescence values of the in vitro cultures and among groups for the in vivo studies were analyzed with a statistical software program (GraphPad Prism version 5.0 for Windows; GraphPad Software, Inc., San Diego, CA, USA), using an independent Student's *t*-test and one-way analysis of variance (ANOVA). A *P*-value of <0.05 was considered statistically significant. The regrowth of the parasite in the viability test was determined based on the statistically significant differences between drug-treated and positive control groups.[9]

Results

In vitro inhibitory effect against *B. divergens*

The in vitro growth of *B. divergens* was significantly inhibited ($P < 0.05$) by 500 nM and 50, 10, 50, and 400 µM treatments of enrofloxacin, enoxacin, trovafloxacin, norfloxacin, and ofloxacin, respectively (Figure 2). Likewise, 500 nM treatments with DA or chloroquine diphosphate significantly inhibited ($P < 0.05$) the in vitro growth of *B. divergens* (data not shown). Enrofloxacin, trovafloxacin, and enoxacin exhibited the highest activity against the in vitro growth of *B. divergens*, followed by norfloxacin and ofloxacin (Table 1). Subsequent viability tests showed that there was no regrowth of the parasite with 50 µM enrofloxacin and trovafloxacin (Table 2). Additionally, treatment of the *B. divergens* in vitro culture with 100 µM enoxacin or tetracycline hydrochloride prevented parasite regrowth (Table 2). Similarly, 400 µM norfloxacin and ofloxacin prevented the in vitro regrowth of *B. divergens* (Table 2). On the other hand, 0.25 µM DA and 0.5 µM chloroquine diphosphate were sufficient to inhibit the regrowth of *B. divergens* (Table 2).

Drug combination test

Fluoroquinolone antibiotic drugs with potential in vitro antibabesial effects were selected to examine their inhibitory effect when used in combination with DA, luteolin, and PYR. The combined application of fluoroquinolone antibiotics/DA revealed an additive effect in all used
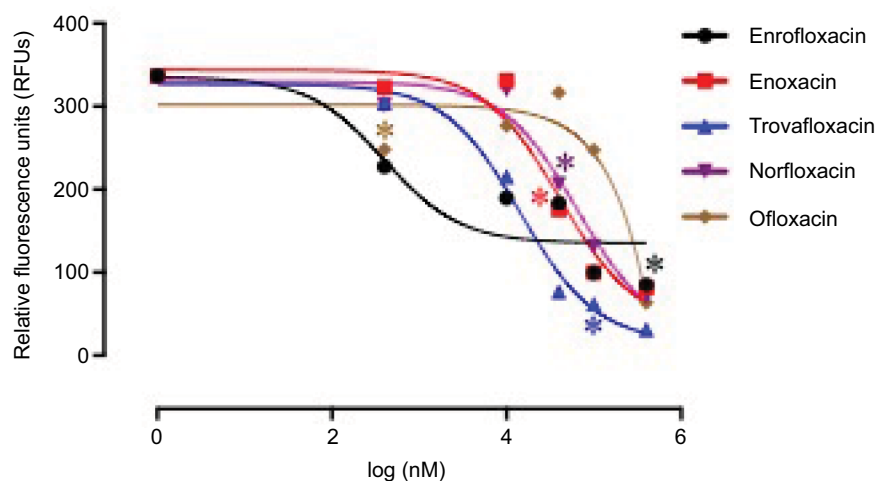

Figure 2 Correlation between relative fluorescence units (RFUs) and the log concentrations of fluoroquinolone antibiotics (nM) in *Babesia divergens* on the fourth day of treatment. Each value represents the mean of triplicate wells after subtraction of the background fluorescence for non-parasitized red blood cells.

Table 1 IC$_{50}$ values of fluoroquinolone antibiotic drugs evaluated for *Babesia divergens* parasite

Drugs	IC$_{50}$ values (µM)[a]
Enrofloxacin	9.54 ± 0.64
Enoxacin	45.23 ± 1.35
Trovafloxacin	13.80 ± 2.69
Norfloxacin	59.52 ± 3.36
Ofloxacin	212.14 ± 3.02
Diminazene aceturate	0.14 ± 0.05
Chloroquine diphosphate	0.40 ± 0.07
Tetracycline hydrochloride	35.27 ± 2.84

Notes: [a]IC$_{50}$ values for each drug were calculated on the fourth day of the in vitro culture using a Babesia fluorescence assay (BFA) in three separate experiments. Each drug concentration was made in triplicate in each experiment, and the final obtained IC$_{50}$ values were the mean ± SD of values obtained from three separate experiments.

Table 2 Viability test results of fluoroquinolone antibiotic drugs evaluated for *Babesia divergens* parasite

Drugs	Drug concentrations (µM)[a]				
	0.5	10	50	100	400
Enrofloxacin	+	+	−	−	−
Enoxacin	+	+	+	−	−
Trovafloxacin	+	+	−	−	−
Norfloxacin	+	+	+	+	−
Ofloxacin	+	+	+	+	−
Tetracycline hydrochloride	+	+	+	−	−
Chloroquine diphosphate	−	−	−	−	−
	Drug concentrations (µM)[a]				
	0.25	0.5	1	5	10
Diminazene aceturate	−	−	−	−	−

Note: [a]Each value was calculated using a Babesia fluorescence assay (BFA) in three separate experiments. Each concentration of the drug was made in triplicate in each experiment. +, viable; −, dead.

Table 3 Synergetic and additive interactions of diminazene aceturate (DA), luteolin, and pyronaridine tetraphosphate (PYR) in combination with enrofloxacin, enoxacin, or trovafloxacin on *Babesia divergens* parasite on the fourth day of treatment

Drug combination	M[a]	FIC$_{D1}$	FIC$_{D2}$	ΣFIC	Degree of interaction[b]
DA$_{D1}$ + enrofloxacin$_{D2}$	M1	0.33	0.24	0.57	Additive
	M2	0.37	0.27	0.64	Additive
	M3	0.42	0.31	0.73	Additive
	M4	0.46	0.34	0.80	Additive
DA$_{D1}$ + enoxacin$_{D2}$	M1	0.33	0.26	0.59	Additive
	M2	0.35	0.27	0.62	Additive
	M3	0.37	0.29	0.66	Additive
	M4	0.47	0.36	0.83	Additive
DA$_{D1}$ + trovafloxacin$_{D2}$	M1	0.09	0.18	0.27	Synergetic
	M2	0.20	0.40	0.60	Additive
	M3	0.25	0.50	0.75	Additive
	M4	0.28	0.57	0.85	Additive
Luteolin$_{D1}$ + enoxacin$_{D2}$	M1	0.39	0.55	0.94	Additive
PYR$_{D1}$ + trovafloxacin$_{D2}$	M1	0.04	0.07	0.11	Synergetic
	M2	0.09	0.13	0.22	Synergetic
	M3	0.26	0.39	0.65	Additive

Notes: [a]M1–M4 refer to the combinations of fluoroquinolone antibiotic drugs combined with different antibabesial drugs; [b]the degree of drug interaction was determined based on the following fractional inhibitory concentration (FIC) index: ≤0.5 (synergetic), >0.5–1 (additive), >1 to <2 (indifferent), and ≥2 (antagonistic). FIC$_{D1}$ refers to the fractional inhibitory concentration of DA, luteolin, or PYR. FIC$_{D2}$ refers to the fractional inhibitory concentration of fluoroquinolone.

combinations, except with the combination M1 for trovafloxacin and DA where IC$_{50}$ value was three-fourth the IC$_{50}$ values of both drugs, that is, a synergetic effect was observed (Table 3). Similarly, treatment of the *B. divergens* parasite with combined therapies consisting of trovafloxacin and PYR significantly enhanced the growth inhibition with a synergetic effect, even at the M2 concentration that consisted of three-fourth the IC$_{50}$ of trovafloxacin and one-half the IC$_{50}$ of PYR (Table 3). The other combinations of either fluoroquinolone antibiotics/PYR or fluoroquinolone antibiotics/luteolin exhibited additive or indifferent drug interactions (Tables 3 and S2).

In vivo effect of fluoroquinolone antibiotics on *B. microti* infection

To further validate fluoroquinolone antibiotics as antibabesial drugs, an in vivo study was performed using *B. microti* in a mouse model in two separate trials. Fluorescence values were significantly inhibited ($P < 0.05$) in mice treated with DA combined with enoxacin from days 4 to 16 pi as compared to those of the controls (Figure 3), while treatment with either enoxacin alone (100 mg·kg^{-1}) or norfloxacin (150 mg·kg^{-1}) produced significant inhibition of fluorescence values ($P < 0.05$) from days 4 to 12 pi as compared to those of the controls (Figure 3). The fluorescence values obtained from mice treated with 700 mg·kg^{-1} ofloxacin were significantly inhibited ($P < 0.05$) from 6 to 16 days pi as compared to the control mice (Figure 3).

Control mice treated with DDW and mice treated with either DA alone or DA combined with enoxacin showed peak fluorescence signals at day 6 pi, whereas other treated mice exhibited peak fluorescence signals at day 8 pi (Figure 3). Notably, mice treated with a combined formula (enoxacin/DA) exhibited significantly inhibited fluorescence values similar to those obtained from mice treated with 25 mg·kg^{-1} DA (Figure 3). These results indicate that a combination formula from DA and enoxacin may be a promising treatment for *Babesia* spp. that infect humans or animals.

Role of fluoroquinolone antibiotics in the regression of anemia in treated mice

The role of fluoroquinolone antibiotics in mouse recovery from anemia was evaluated by monitoring changes in
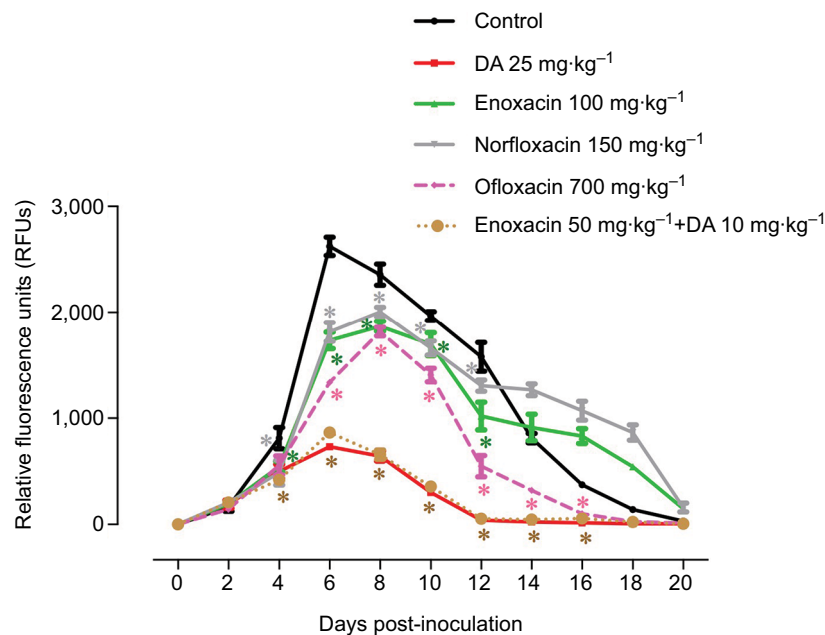
Figure 3 Inhibitory effect of fluoroquinolone antibiotics, diminazene aceturate (DA), and the combination of enoxacin and DA on the growth of *Babesia microti*. Each value represents the mean ± SD of five mice per experimental group. Asterisks indicate significant differences (*$P < 0.05$) from day 4 to day 16 post-inoculation between the fluoroquinolone antibiotic-treated and control groups.

hematological variables. The mice treated with combination therapy consisting of enoxacin and DA exhibited quick recovery from hemolytic and macrocytic anemia associated with babesiosis (Figures 4 and 5). These findings confirm the potential antibabesial effect of this combination therapy.

Detection of *B. microti* nucleic acid remnants in the different organs of treated mice

A nested PCR was applied to DNA samples extracted from the different organs of treated mice on day 20 pi. PCR amplification detected the *B. microti ss-rRNA* gene in the spleen, heart, lungs, and kidneys of mice treated with DDW or DA (Figure 6). Interestingly, parasite nucleic acid was not detected in the lungs of mice treated with an enoxacin and DA combination, while the *B. microti ss-rRNA* gene was detected in other organs (Figure 6).

Discussion

Fluoroquinolones were proven to have antimalarial activity in vitro[13] and in vivo.[14] Considering the close biological similarities between *Plasmodium* and *Babesia* parasites, there is a robust motive for studying the chemotherapeutic potential of fluoroquinolones for their inhibitory effect on babesiosis in vitro and in vivo.

In the current study, the IC_{50} value of trovafloxacin for *B. divergens* was lower than those obtained against chloroquine-sensitive (3D7) and chloroquine-resistant (NF54-R) strains of *Plasmodium falciparum* in vitro.[15] On the other hand, the norfloxacin IC_{50} value for *B. divergens* was higher than those obtained against chloroquine-sensitive (3D7) and chloroquine-resistant (NF54-R) strains of *P. falciparum* in vitro.[15] The enoxacin and ofloxacin IC_{50} values for *B. divergens* were lower than those obtained against a chloroquine-sensitive (3D7) strain of *P. falciparum* in vitro.[15] However, both drugs exhibited IC_{50} values for *B. divergens* higher than those obtained against a chloroquine-resistant (NF54-R) strain of *P. falciparum* in vitro.[15] The IC_{50} of enoxacin for *B. divergens* was higher than those previously calculated against *Babesia bovis*, *Babesia bigemina*, *Theileria equi*, and *Babesia caballi*,[16] which might be attributed to the enoxacin resistance of the *B. divergens* strain (Germany strain) used. Enrofloxacin, enoxacin, trovafloxacin, and norfloxacin exhibited lower IC_{50} values for *B. divergens* than those obtained with N-acetyl-L-cysteine.[17] On the other hand, fluoroquinolone antibiotics exhibited higher IC_{50} values for *B. divergens* than those obtained with PYR, luteolin, and actinonin.[9]

The in vivo evaluation of fluoroquinolone against *B. microti* in mice demonstrated that 100 mg·kg⁻¹ enoxacin, 150 mg·kg⁻¹ norfloxacin, and 700 mg·kg⁻¹ ofloxacin caused 34%, 31%, and 49% inhibition rates, respectively, at day 6
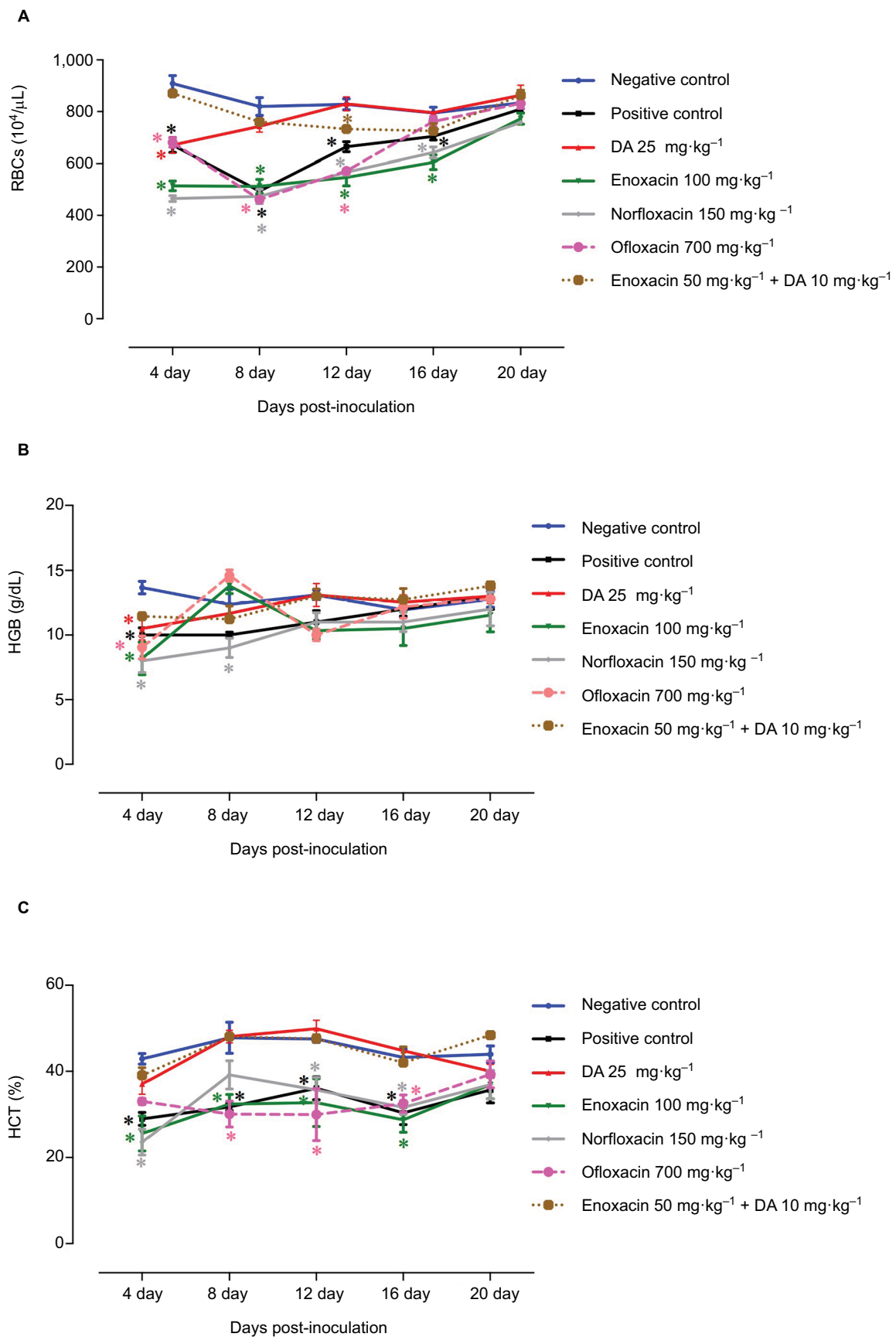
Figure 4 Anemia monitoring in mice treated with fluoroquinolone antibiotics: (**A**) RBCs, (**B**) hemoglobin (HGB), and (**C**) hematocrit (HCT). Each value represents the mean ± SD of five mice per experimental group. Asterisks indicate a significant difference (*$P < 0.05$) between the treated or infected mice and the uninfected mice.
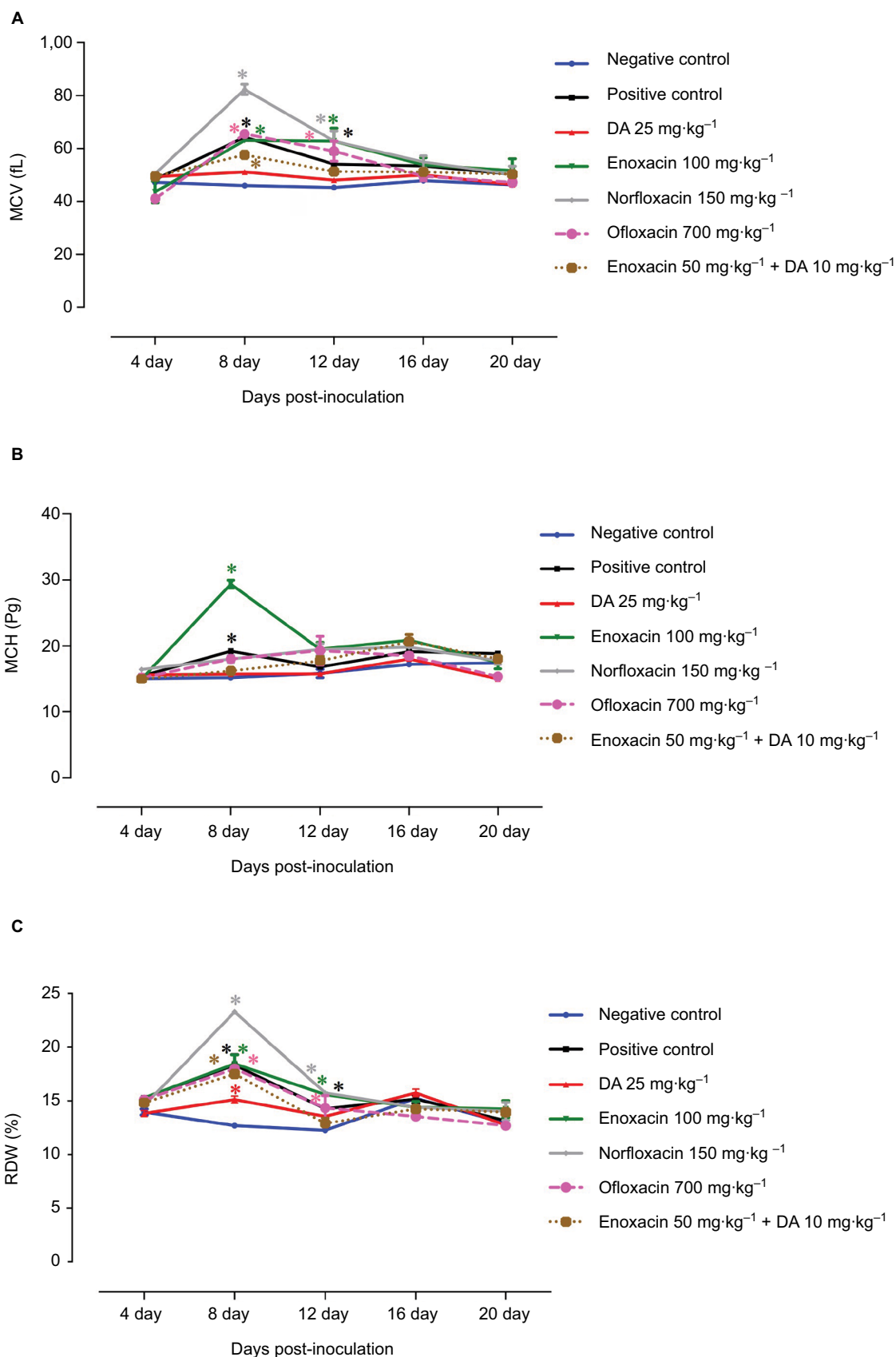**Abbreviation:** DA, diminazene aceturate.

Figure 5 Changes in mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and red blood cell distribution width (RDW) in mice treated with fluoroquinolone antibiotics: (A) MCV, (B) MCH, and (C) RDW. Each value represents the mean ± SD of five mice per experimental group. Asterisks indicate a significant difference (*$P < 0.05$) between the treated or infected mice and the uninfected mice.
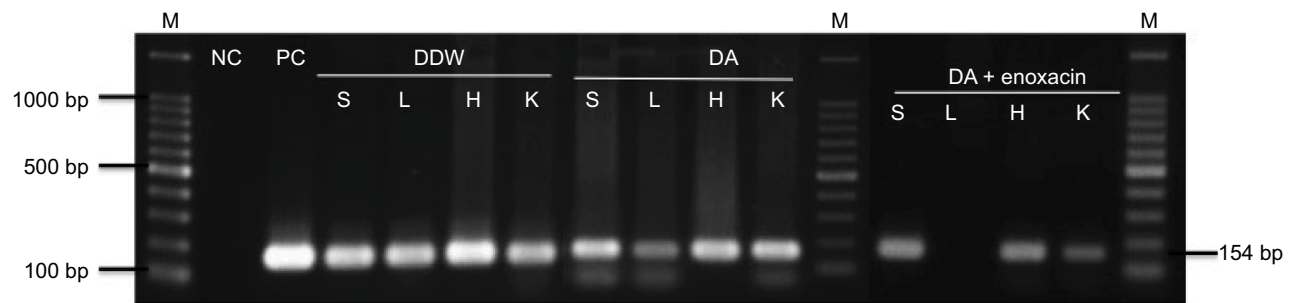**Abbreviation:** DA, diminazene aceturate.

Figure 6 PCR of the *ss-rRNA* gene in different organs of *Babesia microti*-infected mice treated with DDW (positive control), 25 mg·kg⁻¹ diminazene aceturate (DA), and DA (10 mg·kg⁻¹) combined with enoxacin (50 mg·kg⁻¹).
Note: M indicates a 100 bp DNA ladder.
Abbreviations: NC, negative control; PC, positive control; DDW, double-distilled water; S, spleen; L, lung; H, heart; K, kidney; DA, diminazene aceturate; PCR, polymerase chain reaction.

pi. Eight days pi, the inhibition rates caused by 100 mg·kg⁻¹ enoxacin, 150 mg kg⁻¹ norfloxacin, and 700 mg·kg⁻¹ ofloxacin were 21%, 15%, and 23%, respectively. These results are lower than the 77.5% inhibition for 500 mg·kg⁻¹ thiostrepton, 68.5% for 500 mg·kg⁻¹ clindamycin, and 58.3% and 72.4% for 30 and 100 mg·kg⁻¹ allicin, respectively.[11,18]

Enoxacin is a broad-spectrum antibacterial agent used to treat a wide variety of infections, including gastroenteritis, respiratory tract infections,[19] and urinary tract infections.[20] The drug is also characterized by its rapid and thorough absorption from the gastrointestinal tract after administration, with wide distribution throughout the body.[20,21] Of note, the tissue concentrations of enoxacin usually exceed its serum concentrations, and the serum-elimination half-life of this antibacterial agent with normal renal function is ~6 hours,[21] a fact that ensures the continuous effect of the drug over time. Therefore, combination therapy consisting of low doses of enoxacin and a commonly used antibabesial agent such as DA was tested in mice infected by the *B. microti* parasite. The results revealed that an enoxacin/DA combination seems to be more effective than fluoroquinolones alone, even though treatment with reduced doses of both chemicals was administered. A combination of 50 mg·kg⁻¹ enoxacin and 10 mg·kg⁻¹ DA resulted in 67% inhibition at day 6, as compared with 72% inhibition in the presence of a standard dose (25 mg·kg⁻¹) of DA at day 6 pi. However, at day 8 pi, the combined therapy caused a 72% inhibition rate, whereas 25 mg·kg⁻¹ DA caused a 72.5% inhibition rate. These results are higher than the 70% inhibition rates for clindamycin combined with the natural product quinine[22] and the 56.35% and 53.25% inhibition rates for 85 mg·kg⁻¹ PYR combined with 10 mg·kg⁻¹ DA.[10]

Mice treated either with DA alone or with lower doses of an enoxacin and DA combination exhibited quick recovery from this anemia, indicating the potential role of this combination therapy in recovery from the hemolytic macrocytic anemia caused by babesiosis. Moreover, insignificant emitted fluorescence signals were obtained from enoxacin/DA-treated mice on day 20 pi, and the *B. microti ss-rRNA* gene was not detected in the lungs of mice treated with the enoxacin/DA combination. However, a remnant of the parasite's nucleic acid was detected in the lungs of mice treated with DA alone, indicating the promising antibabesial effect of such a combination. Nevertheless, parasite DNA was detected in other organs examined via PCR. The short period of the experiment (20 days) may explain the presence of DNA remnants in mouse organs. Therefore, further studies are required to evaluate the inhibitory effect of enoxacin in combination with other antibabesial drugs such as clofazimine,[23] PYR, or luteolin[9,10,16] against the growth of *Babesia* and *Theileria* parasites for more prolonged periods of infection. Furthermore, future studies are warranted to explore why the DA and enoxacin combination therapy cleared the infection from the lungs of *B. microti*-infected mice, while the parasite DNA was still detected in the other organs of infected mice using a PCR assay.

Currently, the treatment of human babesiosis depends on the administration of combination therapies, such as azithromycin with atovaquone or clindamycin with quinine. However, a failure to recover is reported in some severe cases with the development of drug-resistant parasites.[3] Therefore, the development of novel drugs and a combination formula for treating the infection is required. Safe and cheap fluoroquinolones, such as enoxacin, might be an alternative chemotherapeutic agent against human babesiosis caused by *B. microti* or *B. divergens*.

Limitations
The limitations of this study should be mentioned. In the present study, the inhibitory effects of selected fluoroquinolones against the in vitro growth of *Babesia* parasites were examined when used in combination with DA, luteolin, and PYR. The

synergetic or antagonistic relationships between these antibabesial drugs and either fluoroquinolones (norfloxacin and ofloxacin) or lower doses of currently approved antibabesial drugs in humans, such as azithromycin, were not evaluated against the growth of *B. microti* in mice. Future studies are warranted to evaluate the potential of these combinations for *B. microti*- and/or *B. rodhaini*-infected mice. Successful application of this combination could reduce the potential side effect of currently used antibabesials, such as the cardiotoxicity of azithromycin.

Conclusion

In this study, the inhibitory effects of fluoroquinolones have been demonstrated against *B. divergens* in vitro and against *B. microti* in mice. The results revealed that enoxacin, enrofloxacin, and trovafloxacin exhibited the highest activity against the in vitro growth of *B. divergens*, while enoxacin/DA combination therapy exhibited a potential antibabesial effect in *B. microti*-infected mice. Such findings suggest that an enoxacin/DA combination formula might be useful for the treatment of infection caused by *Babesia* spp.

Data sharing statement

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Acknowledgments

Author contributions

Conceived and designed the experiments: MAR, MA, SAE-SE-S, II. Performed the experiments: MAR, MA, SAE-SE-S. Analyzed the data: MAR, AG. Contributed reagents/materials/analysis tools: MAR, NY, II. Wrote the manuscript: MAR, II. All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

Table S1 Concentrations of fluoroquinolone antibiotic drugs combined with diminazene aceturate, luteolin, and pyronaridine tetraphosphate applied to the cultures of *Babesia divergens* parasite

M[a]	IC$_{50}$[b]			
	Fluoroquinolone antibiotic drugs	Diminazene aceturate	Luteolin	Pyronaridine tetraphosphate
M1	0.75	0.75	0.75	0.75
M2	0.75	0.5	0.5	0.5
M3	0.5	0.75	0.75	0.75
M4	0.5	0.5	0.5	0.5

Note: [a]M1–M4 refer to the combinations of fluoroquinolone antibiotic drugs combined with different antibabesial drugs; [b]combinations were based on the calculated IC$_{50}$ values obtained from the in vitro fluorescence-based assay.

Table S2 Indifferent interaction of diminazene aceturate (DA), luteolin, and pyronaridine tetraphosphate (PYR) in combination with enrofloxacin, enoxacin, or trovafloxacin on *Babesia divergens* parasite on the fourth day of treatment

Drug combination	M[a]	FIC$_{D1}$	FIC$_{D2}$	ΣFIC	Degree of interaction[b]
Luteolin$_{D1}$ + enrofloxacin$_{D2}$	M1	0.89	0.82	1.71	Indifferent
	M2	0.92	0.85	1.77	Indifferent
	M3	0.94	0.87	1.81	Indifferent
	M4	0.96	0.88	1.84	Indifferent
Luteolin$_{D1}$ + enoxacin$_{D2}$	M2	0.48	0.68	1.16	Indifferent
	M3	0.50	0.71	1.21	Indifferent
	M4	0.55	0.78	1.33	Indifferent
Luteolin$_{D1}$ + trovafloxacin$_{D2}$	M1	0.79	0.98	1.77	Indifferent
	M2	0.78	0.96	1.74	Indifferent
	M3	0.78	0.96	1.74	Indifferent
	M4	0.58	0.71	1.29	Indifferent
PYR$_{D1}$ + enrofloxacin$_{D2}$	M1	0.89	0.85	1.74	Indifferent
	M2	0.90	0.86	1.76	Indifferent
	M3	0.94	0.90	1.84	Indifferent
	M4	0.95	0.91	1.86	Indifferent
PYR$_{D1}$ + enoxacin$_{D2}$	M1	0.63	0.69	1.32	Indifferent
	M2	0.68	0.74	1.42	Indifferent
	M3	0.80	0.86	1.66	Indifferent
	M4	0.89	0.97	1.86	Indifferent
PYR$_{D1}$ + trovafloxacin$_{D2}$	M4	0.67	0.98	1.65	Indifferent

Notes: [a]M1–M4 refer to the combinations of fluoroquinolone antibiotic drugs combined with different antibabesial drugs; [b]the degree of drug interaction was determined based on the following fractional inhibitory concentration (FIC) index: ≤0.5 (synergism), >0.5–1 (additive), >1 to <2 (indifferent), and ≥2 (antagonism). FIC$_{D1}$ refers to the fractional inhibitory concentration of DA, luteolin, or PYR. FIC$_{D2}$ refers to the fractional inhibitory concentration of fluoroquinolone drug.