IN VITRO TESTING OF RIFAMPICIN AND FOSMIDOMYCIN FOR ANTI-PARASITIC ACTIVITY AGAINST *THEILERIA EQUI* AND *BABESIA CABALLI*

An Undergraduate Research Scholars Thesis

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TABLE OF CONTENTS

ABSTRACT

In Vitro Testing of Rifampicin and Fosmidomycin for Anti-Parasitic Activity against *Theileria equi* and *Babesia caballi*. (May 2013)

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Equine Piroplasmosis (EP) is a disease that negatively impacts the horse industry worldwide and is caused by two distinct species of protozoan parasites, *Babesia caballi* and *Theileria equi*. Currently the United States is considered free of EP and strict regulations are in place to prevent its introduction. If an outbreak does occur, there are two options for infected horses: lifelong quarantine or humane euthanization. Treatment options are limited in their ability to clear infection. Rifampicin and fosmidomycin are anti-microbial agents that are successful at treating related protozoan diseases, such as malaria. These two drugs were screened for anti-protozoal properties against the erythrocytic stage of *T. equi* and *B. caballi* by performing *in vitro* inhibition assays. Parasite growth was evaluated over a 72 h treatment period and was significantly reduced for both species with notable morphological changes. Rifampicin was shown to possess more potent effects at lower concentrations than fosmidomycin for both *T. equi*

and *B. caballi*. Based on data acquired during the trial, further *in vitro* and *in vivo* tests should be performed for rifampicin and fosmidomycin. Further, the results attained suggest that other existing anti-microbials should be evaluated for their efficacy in treating EP.

DEDICATION

I would like to dedicate this senior scholar's thesis to my mother, Dee Anna Cobb, and my sister, Lindsay Cobb who have always taught me to follow my dreams. Without their support I would not be where I am today. I would further like to dedicate this thesis to my girlfriend, Estefanía Ordaz, who has been my constant companion and voice of reason throughout my college career.

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NOMENCLATURE

cELISA	Competitive Enzyme-Linked Immunosorbent Assay
CFT	Complement Fixation Test
DOXP	2-*C*-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate
DXP	1-deoxy-D-xylulose 5-phosphate
EP	Equine Piroplasmosis
IFA	Immunofluorescence Assay
PCR	Polymerase Chain Reaction
RBC	Red Blood Cells
USDA	United States Department of Agriculture

CHAPTER I

INTRODUCTION

Equine Piroplasmosis (EP) is a disease in equids caused by one of two piroplasms: *Babesia caballi* or *Theileria equi*. These blood-borne parasites are transmitted by ticks to their host during feeding (Levine 1985). Piroplasms, of the order Piroplasmida, are members of a phylum of organisms that are obligate parasites called Apicomplexa (Allsopp *et al.*, 1994; Wiser, 2012). The most striking feature of piroplasms is that they are obligate intraerythrocytic organisms of several genera, including *Babesia* and *Theileria* (Levine, 1985; Lack *et al.*, 2012). Two other important features of these organisms are the presence of an organelle called an apicoplast which plays a role in the metabolic functioning of these single-celled organisms with notable similarities to that of the chloroplast in plants (Seeber and Soldati-Favre, 2010) and the presence of an apical complex which plays a role in host cell invasion and attachment (Tonkin, 2011).

EP is enzootic in many regions in the world, including portions of Africa, Europe, Eurasia, the Caribbean and Central and South America (Schein, 1988; de Waal, 1992; Friedhoff and Soulé, 1996; Rothschild and Knowles, 2007). The pathology of the disease is dependent on the severity of infection with acute cases presenting with fever, anemia, icterus, swollen abdomen, and belabored breathing. Death is not uncommon. In less severe disease, the clinical signs are weakness and a lack of appetite.

EP is a disease of worldwide importance with negative implications on trade, importation, and exportation of equids. EP is a reportable disease as defined by the World Organization for Animal Health (Katz *et al.*, 1999). Specifically, international horse trade is impacted in that, for

many countries, negative test results for EP must be confirmed before a horse may enter an EP non-endemic country. The United States has been declared free of EP since 1988 as a result of a joint eradication program put in place by the United States Department of Agriculture (USDA) and the Animal and Plant Health Inspection Services (APHIS), and a Florida State program specifically to eradicate *B. caballi* that began in 1962 (Brüning, 1996). However, due to the fact that the United States shares a land border with Mexico, an EP enzootic country, and the United States is a natural habitat of competent tick vectors for EP, the threat of reintroduction of the disease is constant (George *et al.*, 2002).

Prior to 1901, EP was called by many other names such as anthrax fever, bilious fever, and equine malaria (Roberts *et al.*, 1962). The name equine malaria was used because many of clinical signs of the disease are shared between malaria and EP. In 1901, the intra-erythrocytic forms of the causative agents of EP were examined morphologically and named *Piroplasma equi* due to the fact that the protozoans assume a pear-shape that is characteristic of piroplasms (Laveran, 1901). South African veterinarian Sir Arnold Theiler pioneered research on this disease trying unsuccessfully to infect unaffected horses via blood transfusions from afflicted animals. He cited his inability to initiate disease in the unaffected horses as most likely due to one of two reasons: either the horses were already immune to the infection or because a vector was needed for transmission, namely a tick (Theiler, 1902). Later, in 1912, it was discovered that EP was caused by two distinct organisms, *Babesia equi* (AKA *Piroplasma equi*) and *Babesia caballi* (Nuttall and Strickland, 1912). In 1998 Mehlhorn and Schein re-described *B. equi* as *Theileria equi* due to the fact that before *T. equi* invades the host erythrocyte, it first replicates inside a lymphocyte while *Babesia sensu stricto* parasites only replicate inside the erythrocytes.

Another differentiating factor is that *T. equi* can only be passed transstadially in the tick vector while *Babesia* parasites are passed both transstadially and transovarially (Mehlhorn and Schein, 1998). While there has been recent investigation as to whether *T. equi* should be considered a protozoan of the genus *Theileria* or rather of *Babesia* as previously (Traub-Dargatz *et al.*, 2010), a definitive conclusion has not been reached and for the remainder of this thesis the name *Theileria equi (T. equi)* will be used.

One of the hallmark methods of diagnosing this disease has been and remains examination of stained thin blood smears. The characteristic morphological aspects of *T. equi* and *B. caballi* make for a very confirmatory diagnosis upon seeing the infected erythrocytes of the equid in instances of high parasitemia (de Waal, 1992). *T. equi* and *B. caballi* are morphologically distinct from each other in that *B. caballi* reproduces by forming two paired parasites inside one erythrocyte while *T. equi* forms a tetrad. The divided forms of *B. caballi* are larger than *T. equi* as well (Mehlhorn and Schein, 1998). Definitive diagnosis is based on the divided forms with visualization of tetrads confirming infection with *T. equi* and paired piroplasms confirming infection with *B. caballi.*

However, there are several other methods based on serology for reaching a diagnosis of EP. The complement fixation test (CFT) was developed in 1945 by Hirato *et al*. It is also suggested that the CFT be used in combination with immunofluorescence assay (IFA). Together these two tests can give a confirmatory diagnosis of EP (Weiland, 1986). Also, a competitive enzyme-linked immunosorbent assay (cELISA) was developed by a group of researchers at Washington State University and the USDA's Agricultural Research Service, and is now the official test that is

used in the United States for regulatory testing and detection of EP (Katz *et al.*, 2003). Polymerase Chain Reaction (PCR) protocols have also been developed and are highly sensitive and specific to the detection of both these parasites (Possnet and Ambrosio, 1991; Possnet *et al.*, 1991).

Previously the only confirmed natural vector of EP present in the United States was the tick *Dermacentor (Anocenter) nitens* which transmits *B. caballi* (Drummond *et al.*, 1969). However, under experimental conditions it now has been shown that several other tick species are possible competent vectors of these agents in the United States. Both *Dermacentor variabilis* and *Dermacentor albipictus* have been shown experimentally to be competent vectors of *B. caballi* while *D. variabilis, Rhipicephalus (Boophilus) microplus,* and *Amblyomma cajennense* have been shown experimentally to be competent vectors of *T. equi* (Stiller *et al.*, 1980; Stiller *et al.*, 2002; Scoles *et al.*, 2011). This is important to note due to the fact that all of the ticks except *R. microplus* are found in the United States naturally.

It should be noted that *R. microplus,* along with *Rhipicephalus annulatus,* was eradicated from the United States in 1943 due to efforts to rid the country of the disease bovine babesiosis, which cost the United States large economic losses yearly (Graham and Hourrigan, 1977). The United States is officially free of these ticks even though occasional outbreaks do occur. This is due to the fact that the southern United States, principally Texas, shares a long land border with Mexico where the ticks in question are abundant. Whenever an outbreak does occur, wide scale efforts are put in to place to control the spread via heavy quarantining and other methods. Since 1943 Texas has had a permanent quarantine zone along the Rio Grande bordering Mexico, which is

meant to serve as a barrier to re-infestation with *R. microplus* and *R. annulatus* (Graham and Hourrigan, 1977). However, a recent study has shown that the presence of *R. microplus* may be increasing in south Texas (Lohmeyer *et al.*, 2011).

The life cycles of the causative parasites of EP are similar. In the host *B. caballi* sporozoites introduced at tick feeding invade erythrocytes where they then develop into trophozoites. The trophozoites then grow and divide forming two merozoites. The merozoites egress from the cell and are then capable of infecting more erythrocytes where more replication will occur. The merozoites also can differentiate into gametocytes which, when ingested by the tick vector, undergo a sexual stage and then multiply in various tick tissues, including the salivary glands and the ovaries (Levine, 1985). The most notable difference for *T. equi* is that initially the sporozoites invade lymphatic cells forming schizonts. The schizonts differentiate into merozoites which egress from the cell and then invade erythrocytes. The merozoites then grow and transform into trophozoites which divide into a pear-shaped piroplasms in a tetrad formation. Again, the merozoites can differentiate into gametocytes which, when ingested by the vector, undergo development within the tick culminating in infective sporozoites in the salivary glands, but not in the ovaries and developing eggs (Zapf and Schein, 1994).

Inside the vector tick, again *T. equi* and *B. caballi* differ in their transmission mechanisms. *B. caballi* is transmitted both transstadially and transovarially while *T. equi* is only passed transstadially (Ali *et al.,* 1996). Transstadial transmission occurs when the parasite is passed between separate life stages of the tick vector. Transovarial transmission occurs when the parasite is passed from the infected tick to the eggs of the next generation.

D. variabilis and *A. cajennense* are three-host ticks meaning they feed on three separate hosts during their life cycle, while *D. albipictus*, *D. nitens*, and *R. microplus* are one host ticks meaning they feed on only one host during their 3-stage (larva, nymph, adult) life cycle (Oliver *et al.*, 1987; Scoles *et al.*, 2011). The distribution of the vector ticks is as follows: *R. microplus* is considered to be only present in the quarantine zone along the Texas/Mexico land border, *D. nitens* is found in south Texas and southern Florida, *D. variabilis* and *D. albipictus* are widely distributed across the United States, and *A. cajennense* is only present in southern United States (Estrada-Peña *et al.*, 2004).

Clearing of the agents of EP from an infected animal presents a challenging problem, especially for *T. equi*, due to the lack of effective drugs. A recent study by Schwint *et al.* (2009) demonstrated that treatment with imidocarb dipropionate is effective at clearing an infection of *B. caballi*. However, an earlier study showed that repeated high-dosage treatment with the same drug was ineffective (Butler *et al.*, 2008). In general, it is accepted that imidocarb dipropionate is a valid treatment option for clearing infection with *B. caballi* (Correa *et al.*, 2005). However the same cannot be said for *T. equi*. Treatment with imidocarb dipropionate will not clear the infection (Correa *et al.*, 2005; Kumar *et al.*, 2003). Infection with *T. equi*, as far as is known, is not able to be cleared under any current treatment protocols. Several drugs (triclosan, artesunate, pyrimethamine, pamaquine, ponazuril, artemisinin derivatives, or buparvaquone) have shown promise with both theilerial-static and theilerial-cidal activities *in vitro* (Bork *et al.*, 2003; Nagai *et al.*, 2003; Wise *et al.*, 2011). However, due to a lack of *in vivo* trials and pharmacokinetic studies on these drugs, further research must be done to investigate their efficacy against *T. equi*.

Because of the lack of effective clearing agents, specifically for infection with *T. equi*, this study evaluated the *in vitro* effectiveness of two candidate drugs. The two candidate drugs were tested alongside a positive control drug, diminazene aceturate. Diminazene aceturate is considered a positive control due to its proven *in vitro* activity against *T. equi* and *B. caballi*, but it produces considerable adverse effects in the horse (Vial and Gorenflot, 2006). The two candidate drugs are fosmidomycin and rifampicin.

Fosmidomycin is an antibiotic that is known to inhibit the non-mevalonate pathway or 2-*C*-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway (DOXP) pathway present in protozoans of the phylum Apicomplexa, of which *T. equi* and *B. caballi are* members (Kuzuyama *et al.*, 1998; Kuzuyama *et al.*, 2000). It inhibits the action of 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXP reductoisomerase), an enzyme of the DOXP pathway (Kuzuyama *et al.*, 1998).

Rifampicin is thought to act by inhibiting the DNA-dependent RNA polymerase present in prokaryotes (Strath *et al.*, 1993). While *T. equi* and *B. caballi* are eukaryotes rather than prokaryotes, this drug has been shown to inhibit the growth of the closely related apicomplexan parasites *Plasmodium* spp. both *in vitro* and *in vivo* (Strath *et al.*, 1993; Aditya *et al.*, 2010). However, once the drug regimen was discontinued, recrudescence of malaria occurred (Strath *et al.*, 1993). Because *T. equi*, *B. caballi* and *Plasmodium* spp. are closely related piroplasms of the phylum Apicomplexa, it is not unlikely that rifampicin will exhibit anti-theilerial and anti-babesial activity as well.

Taking all pertinent factors into consideration, it is hypothesized that treatment with fosmidomycin will exhibit theilericidal and babesiacidal activity against *T. equi* and *B. caballi*, respectively, while treatment with rifampicin will only exhibit static activity and once the treatment regimen is ceased, recrudescence will occur for both *T. equi* and *B. caballi*.

CHAPTER II

MATERIALS AND METHODS

Equine donor erythrocyte preparation and storage

Donor blood from a 22 year old female Tennessee Walking Horse (*Equus ferrus caballus*) was collected via jugular venipuncture into a 30 mL syringe containing 15 mL of Alsever's solution in compliance with the approved animal use protocol (TAMU AUP 2012-218, PI Dr. Patricia J. Holman). Following blood collection, the blood was transferred to a 50 mL centrifuge tube and centrifuged for 15 min at 330 X g at approximately 10°C and stored at 4°C until further preparation was needed. When donor erythrocytes were required, 1 volume of packed red blood cells (RBC) was added to 7 volumes of Dulbecco's Phosphate Buffered Saline (DPBS) (HyClone Laboratories, Logan, UT) and resuspended thoroughly in a 2 mL microcentrifuge tube. The resuspended erythrocytes were centrifuged in a microcentrifuge at 820 X g for 3 min at room temperature (RT). The supernatant was discarded. This wash procedure was repeated twice more for a total of 3 washes. After the supernatant was removed from the last wash, 5 volumes of Puck's Saline Glucose with 20 g/L extra glucose (PSG+G) (Holman *et al.*, 1988) were added to each microcentrifuge tube which was then centrifuged once more as above and stored at 4°C until use.

Resuscitation of *Babesia caballi* and *Theileria equi* cultures from cryopreserved stocks

Media formulations were as follows. *Theileria equi* HL-20 Complete Media (HL-20 CTE) was composed of 20% fetal bovine serum (V/V; Hyclone Laboratories), 2 mM L-glutamine, 1 mg/mL AlbuMAX I (GIBCO, Grand Island, NY), 16 µM thymidine (HT Supplement;

GIBCO), 100 U/mL penicillin, 100 µg/mL streptomycin per mL, 25 µg/mL amphotericin B (Fungizone) (antibiotic-antimycotic solution, GIBCO), 2% HB 101 supplement (V/V after following manufacturer's instructions on reconstitution of the lyophilized supplement; Irvine Scientific, Santa Ana, CA) in HL-1 medium (Lonza, Walkersville, MD, USA) (Holman *et al.*, 1994). *Babesia caballi* HL-20 Complete Media (HL-20 CBC) was composed of 20% normal adult horse serum (V/V; Atlanta Biologicals, Lawrenceville, GA), 2 mM L-glutamine, and antibiotic-antimycotic as above, in HL-1 medium (Holman *et al.*, 1993). Media were sterile filtered (0.45 micron filter) and stored at 4°C.

Babesia caballi (USDA strain) and *T. equi* (USDA strain) cryostocks were resuscitated from liquid nitrogen storage according to standard protocols (Holman *et al.*, 1993 & 1994). The parasites were cultured in 24-well plates. The total well volume for initiation and maintenance of cultures, 1.25 mL, contained 900 µL media, 100 µL washed donor horse RBC, and 250 µL cryopreserved culture of *T. equi* at passage 18 and *B. caballi* at passage 32, which had been rapidly thawed by swirling the vial in a waterbath at 37°C. Each day, 900 µL of medium overlying the settled RBC layer was removed and replaced with fresh medium. At that time, thin erythrocyte smears were made from 0.5 µL of the *B. caballi* and *T. equi* settled erythrocytes in culture, methanol fixed, and stained for 45 min in a 1:15 Giemsa:H_2O solution (Accustain, Sigma, St. Louis, MO). Following staining, the smears were rinsed with distilled water for 1 min to remove excess stain and debris and allowed to air dry. Examination of the thin erythrocyte films was done at 1000X magnification under oil immersion light microscopy. Before reaching parasitemias suitable for routine subculturing, the entire smear was examined. One hundred microliters of fresh RBC was added to each well on a weekly basis.

Once 7 parasites per 1000X field were seen in at least 2 fields under oil immersion, a subculture was performed by adding to each new well 250 µL culture, 900 µL complete media, and 100 µL washed RBC to achieve a subculture ratio of 1:5 for both *B. caballi* and *T. equi* (passage 33 and 19, respectively). When the cultures consistently maintained at least 7 parasites per 1000X field in at least 2 fields under oil immersion light microscopy, a regular subculturing regimen was adopted. *B. caballi* cultures were subcultured every 72 h and *T. equi* cultures were subcultured every 48 h, with daily replenishing of the medium.

Drug trials

Diminazene aceturate (Sigma-Aldrich, St. Louis, MO), fosmidomycin (Invitrogen, Grand Island, NY), and rifampicin (Sigma, St. Louis, MO) were solubilized to stock concentrations of 50 mM, 50 mM, and 30 mM in ultrapure water containing 10%, 10%, and 50% dimethyl sulfoxide (DMSO) (V/V), respectively. The stock solutions were stored at -20°C until use. At use, the stocks were thawed at RT and an appropriate volume was added to HL-1 medium to yield 0.5 mM working solutions for diminazene aceturate and fosmidomycin, and a 0.3 mM working solution for rifampicin. Concentrations of diminazene aceturate used as positive controls were 2.4 µM and 6.0 µM for *B. caballi* and *T. equi*, respectively. The concentrations of rifampicin and fosmidomycin used in the drug trials are shown in Table 1 and Table 2.

Table 1

Concentrations of rifampicin and fosmidomycin tested for inhibition of *Theileria equi in vitro*

Rifampicin (µM)	Fosmidomycin (µM)
3.75	8
7.5	16
15	32

Table 2

Concentrations of rifampicin and fosmidomycin tested for inhibition of *Babesia caballi in vitro*

Rifampicin (µM)	Fosmidomycin (µM)
3.75	8
7.5	16
15	32

The drug-containing media for the trials were made by adding the appropriate amount of 0.3 mM or 0.5 mM working solution to HL-20 CTE for *T. equi* or HL-20 CBC for *B. caballi.* DMSO control media were prepared by adding an aliquot of 50% solution of DMSO in ultrapure water to HL-20 CTE or HL-20 CBC to mimic the maximum concentration of DMSO in the drug-containing media. Negative control media were HL-20 CBC for *B. caballi* and HL- 20 CTE for *T. equi*.

The cultures were adjusted to obtain a standard starting inoculum parasitemia of 2% as follows. First, hematocrits of regularly maintained *T. equi* and *B. caballi* cultures were determined by resuspending the cultured cells and drawing 30 µL into a mini-hematocrit capillary tube and then sealing the end. Centrifugation in a hematocrit centrifuge followed and the hematocrit was then read according to the manufacturer's instructions (Damon, IEC Division, Needham, MA). Aliquots of 200 µL of culture were centrifuged in a table-top centrifuge at 820 X g for 3 min at RT and thin erythrocyte smears were made from the cell pellets in triplicate and stained as above. To determine the parasitemia of the cultured cells, the number of parasitized and non-parasitized erythrocytes were tallied separately (Fisher Differential Counter Model 111, Fisher Scientific, Pittsburgh, PA) in each of 10 fields of approximately 100 cells each (total of 1000 cells) at 1000X magnification under oil immersion light microscopy. The total number of parasitized RBC was then divided by 1000 and multiplied by 100 to give a percent parasitemia. An average percent parasitemia was calculated from the values of the triplicate smears. Washed donor RBC were prepared as above and the hematocrit adjusted with complete HL-1 medium to that of the culture and then added to the culture at a factor so as to achieve a 2% parasitemia. To confirm the starting parasitemia of 2% for the trial, the hematocrit of the adjusted culture was

then measured as above and triplicate Giemsa-stained thin erythrocyte smears were made and percent parasitemias were calculated as above.

To 12 wells in a 96 well plate 75 µL of the inoculum was plated along with 75 µL of a 2X drug or DMSO-containing media (which resulted in a 1X drug or DMSO concentration), or negative control medium for each drug concentration to be tested. To provide adequate humidity to prevent the wells from drying out, 200 µL of sterile ultrapure water was added to all the outermost wells of the plate. Every 24 h for 3 d 100 µL of medium overlying the settled erythrocytes was removed from each well and replaced with 100 µL fresh 1X drug-containing, negative control, or DMSO medium as appropriate. For each parasite, trials with rifampicin and fosmidomycin were performed in tandem using the same negative, positive, and DMSO control cultures for both drugs. Thin erythrocyte smears were made from 3 separate wells every 24 h and stained and examined as above. The percent parasitemias and average percent parasitemias of the matched triplicate samples were recorded. After 72 h of drug treatment, the medium was replaced with drug-free complete medium. At 96 h, the cultures were evaluated for parasites that appeared viable. If intact, normal-appearing parasites were detected, the cultures were terminated. If not, the cultures were monitored until recrudescence occurred or maintained for an additional 14 d by replenishing with drug-free medium as above.

Based on the trial data, further trials with *T. equi* were performed over appropriate ranges of drug concentrations as shown in Table 3 and Table 4 in the same manner as described.

Table 3

Concentrations of rifampicin and fosmidomycin tested for inhibition of *Theileria equi in vitro*

Rifampicin (µM)	Fosmidomycin (µM)
0.75	1.5
1.5	3
3	6
6	12
12	24

Table 4

Concentrations of rifampicin and fosmidomycin tested for inhibition of *Theileria equi in vitro*

Rifampicin (µM)	Fosmidomycin (µM)
15	32
30	64
60	100

Determination of IC_{50}

The average percent parasitemias at 72 h for each drug concentration and the negative control were input into a Microsoft Excel graph and a trendline was added (either linear or power-based as appropriate to its best fit). Using the equation obtained by this process, the IC_{50} was - calculated and reported.

Data collection and analysis

During trials triplicate percent parasitemias were determined and recorded in Microsoft Excel. For each set of triplicate wells, average percent parasitemia was calculated and tested in Student's t-tests to compare the parasitemias between the trial drug, positive control, and negative control cultures. *P* values of ≤ 0.05 were considered statistically significant.

CHAPTER III

RESULTS

Theileria equi

Initially, rifampicin was tested at 3.75, 7.5, and 15.0 µM against *T. equi* (Table 1, Fig. 1A). The negative control and DMSO-treated cultures showed maximum parasitemias of 23.8% and 22.8% respectively at 72 h as shown in Table 5. These cultures showed no difference in growth statistically ($P > 0.20$). The positive control cultures, which were treated with 6.0 µM diminazene aceturate, reached a maximum parasitemia of 2.6% at 72 h (Table 5, Fig. 1A). This was significantly inhibited when compared with the negative control cultures ($P < 0.001$). The cultures treated with 3.75, 7.5, and 15.0 µM rifampicin reached maximum parasitemias of 13.0%, 11.3%, and 10.5% respectively at 72 h (Table 5). Each of these was statistically significantly inhibited in comparison with the negative control cultures ($P < 0.001$, $P < 0.001$, $P < 0.001$, respectively).

Fosmidomycin was tested at 8.00, 16.0, and 32.0 µM against *T. equi* alongside the rifampicin trial (Fig. 1B). As such, the control data is the same. The cultures treated with 8.0, 16.0, and 32.0 µM fosmidomycin reached maximum parasitemias of 16.0%, 13.5%, and 12.2% respectively at 72 h (Table 5, Fig. 1B). Each of these was inhibited significantly in comparison with the negative control cultures ($P < 0.003$, $P < 0.001$, $P < 0.001$, respectively).

Following this initial trial, a second trial was performed (Fig. 2). Rifampicin was tested at concentrations of 0.75, 1.5, 3.0, 6.0, and 12.0 µM against *T. equi* (Fig. 2A). The negative control

and DMSO- treated cultures reached maximum parasitemias of 21.6% and 20.4% respectively at 72 h as shown in Table 6. There was no statistical difference between the growth rates of these two cultures ($P > 0.30$). *T. equi* control cultures treated with 6.0 µM diminazene aceturate attained a maximum parasitemia of 2.9% (Table 6) at 72 h and were significantly inhibited when compared with the negative control cultures ($P < 0.001$). The cultures treated with 0.75, 1.5, 3.0, 6.0, and 12.0 µM rifampicin reached maximum parasitemias of 16.5%, 15.6%, 13.6%, 12.9%, and 9.6% respectively at 72 h (Table 6). Each of these was significantly inhibited in comparison with the negative control cultures ($P < 0.002$, $P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.001$, respectively). Fosmidomycin was tested at 1.5, 3.0, 6.0, 12.0, and 24.0 µM against *T. equi* (Fig. 2B) alongside the rifampicin trial, so the control culture data is the same for both drugs. Maximum parasitemias of 16.9%, 15.4%, 15.3%, 13.6%, and 12.1% were attained at 72 h for treatment with 1.5, 3.0, 6.0, 12.0, and 24.0 µM fosmidomycin, respectively (Table 6). Each of these cultures was significantly inhibited with comparison to the negative control cultures ($P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.001$, respectively).

In the last trial performed with *T. equi*, rifampicin was tested at 15.0, 30.0, and 60.0 µM (Fig. 3A). Negative control and DMSO-treated cultures reached maximum parasitemias of 19.7% and 19.5% at 72 h (Table 7) with no statistical difference in their growth ($P > 0.50$). Cultures treated with 6.00 µM diminazene aceturate attained a maximum parasitemia of 2.6% at 72 h (Table 7) and were significantly different from the negative control cultures ($P < 0.001$). Maximum parasitemias 10.7%, 9.5%, and 7.5%, respectively, were attained at 72 h (Table 7). Each of these cultures was significantly inhibited compared to the negative control cultures ($P < 0.001$, $P < 0.001$, $P < 0.001$, respectively). Fosmidomycin was tested 32.0, 64.0, and

100.0 µM against *T. equi* (Fig. 3B) alongside the rifampicin trial, so the control culture data is the same for both drugs. Maximum parasitemias of 12.5%, 10.3%, and 8.1% were attained at 72 h for treatment with 32.0, 64.0, and 100.0 µM fosmidomycin, respectively (Table 7). Each of these cultures was significantly inhibited with comparison to the negative control cultures ($P < 0.002$, $P < 0.001$, $P < 0.001$, respectively). Distinct morphological changes were observed in the cultured parasites treated with rifampicin, fosmidomycin and diminazene aceturate in comparison with those that were not treated or treated with DMSO only as shown in Figure 4. In the cultures treated with rifampicin, fosmidomycin, or diminazene aceturate a dot forms of the parasite predominated (Fig. 4).

The IC_{50} determined for *T. equi* treated with rifampicin is 22.86 µM. The equation obtained from the graph in Microsoft Excel is shown below.

Equation 1: $y = 0.0057x^2 - 0.5349x + 19.115$

The R^2 coefficient obtained via this analysis is equal to 0.9471, suggesting a high degree of correlation.

The IC_{50} for *T. equi* treated with fosmidomycin is 58.51 µM. The equation obtained from the graph in Microsoft Excel is shown below.

Equation 2: $y = 0.0012x^2 - 0.2347x + 19.491$

The R^2 coefficient obtained via this analysis is equal to 0.9856, suggesting a high degree of correlation.

Upon removal of drug pressure, parasite growth resumed in all instances for *T. equi* (Table 8).

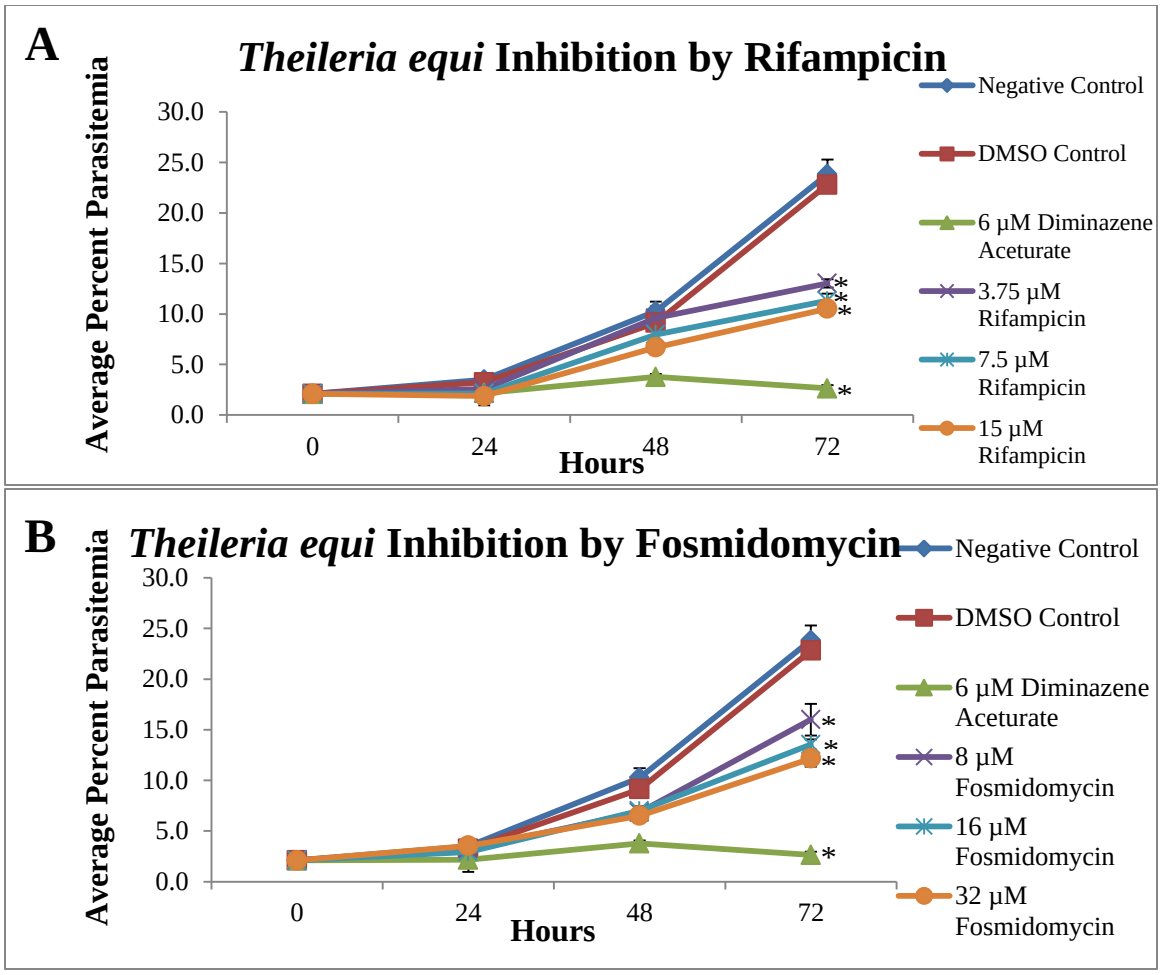

Fig. 1. (A) *In vitro* growth inhibition trials of *T. equi* against a range of concentrations of rifampicin; (B) *In vitro* growth inhibition trials of *T. equi* against a range of concentrations of fosmidomycin. Bars show standard deviations. Points with an asterisk (*) exhibit statistically significant inhibition of growth.

Table 5

Average percent parasitemias of *T. equi* at 72 h treated with rifampicin and fosmidomycin. Cultures treated with complete media, DMSO, and 6.0 µM diminazene aceturate served as controls. *P* values were calculated using a Student's t-test between the 'No Treatment' control and the drug treatment percent parasitemias. *P* values < 0.05 are considered statistically significant.

Treatment	Average Percent Parasitemia	P Value
No Treatment	23.8±1.46	N/A
DMSO	22.8±0.361	> 0.200
6.0 µM Diminazene Aceturate	2.6±0.322	< 0.001
3.75 µM Rifampicin	13.0±0.416	< 0.001
7.5 µM Rifampicin	11.3±0.721	< 0.001
15.0 µM Rifampicin	10.5±0.351	< 0.001
8.0 µM Fosmidomycin	16.0±1.56	< 0.003
16.0 µM Fosmidomycin	13.5±0.503	< 0.001
32.0 µM Fosmidomycin	12.2±0.808	< 0.001

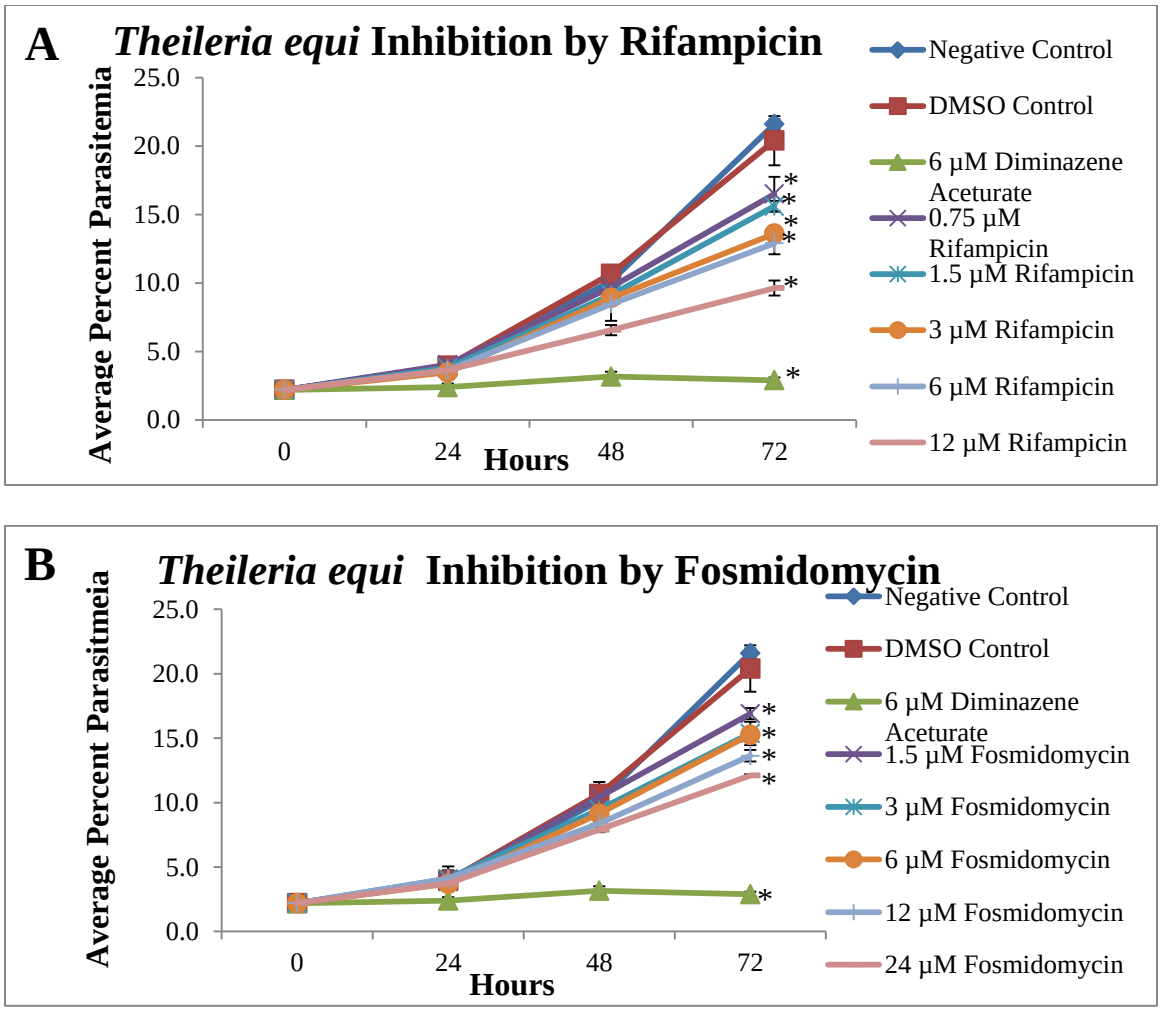

Fig. 2. (A) *In vitro* growth inhibition trials of *T. equi* against a range of concentrations of rifampicin; (B) *In vitro* growth inhibition trials of *T. equi* against a range of concentrations of fosmidomycin. Bars show standard deviations. Points with an asterisk (*) exhibit statistically significant inhibition of growth.

Table 6

Average percent parasitemias of *T. equi* at 72 h treated with rifampicin and fosmidomycin. Cultures treated with complete media, DMSO, and 6.00 µM diminazene aceturate served as controls. *P* values were calculated using a Student's t-test between the 'No Treatment' control and the drug treatment percent parasitemias. *P* values < 0.05 are considered statistically significant.

Treatment	**Average Percent Parasitemia**	***P* Value**
No Treatment	21.6±0.361	N/A
DMSO	20.4±1.80	< 0.300
6.0 µM Diminazene Aceturate	2.9±0.200	< 0.001
0.75 µM Rifampicin	16.5±1.25	< 0.002
1.5 µM Rifampicin	15.6±0.400	< 0.001
3.0 µM Rifampicin	13.6±0.557	< 0.001
6.0 µM Rifampicin	12.9±0.794	< 0.001
12.0 µM Rifampicin	9.6±0.551	< 0.001
1.5 µM Fosmidomycin	16.9±0.436	< 0.001
3.0 µM Fosmidomycin	15.4±0.907	< 0.001
6.0 µM Fosmidomycin	15.3±0.351	< 0.001
12.0 µM Fosmidomycin	13.6±0.451	< 0.001
24.0 µM Fosmidomycin	12.1±0.100	< 0.001

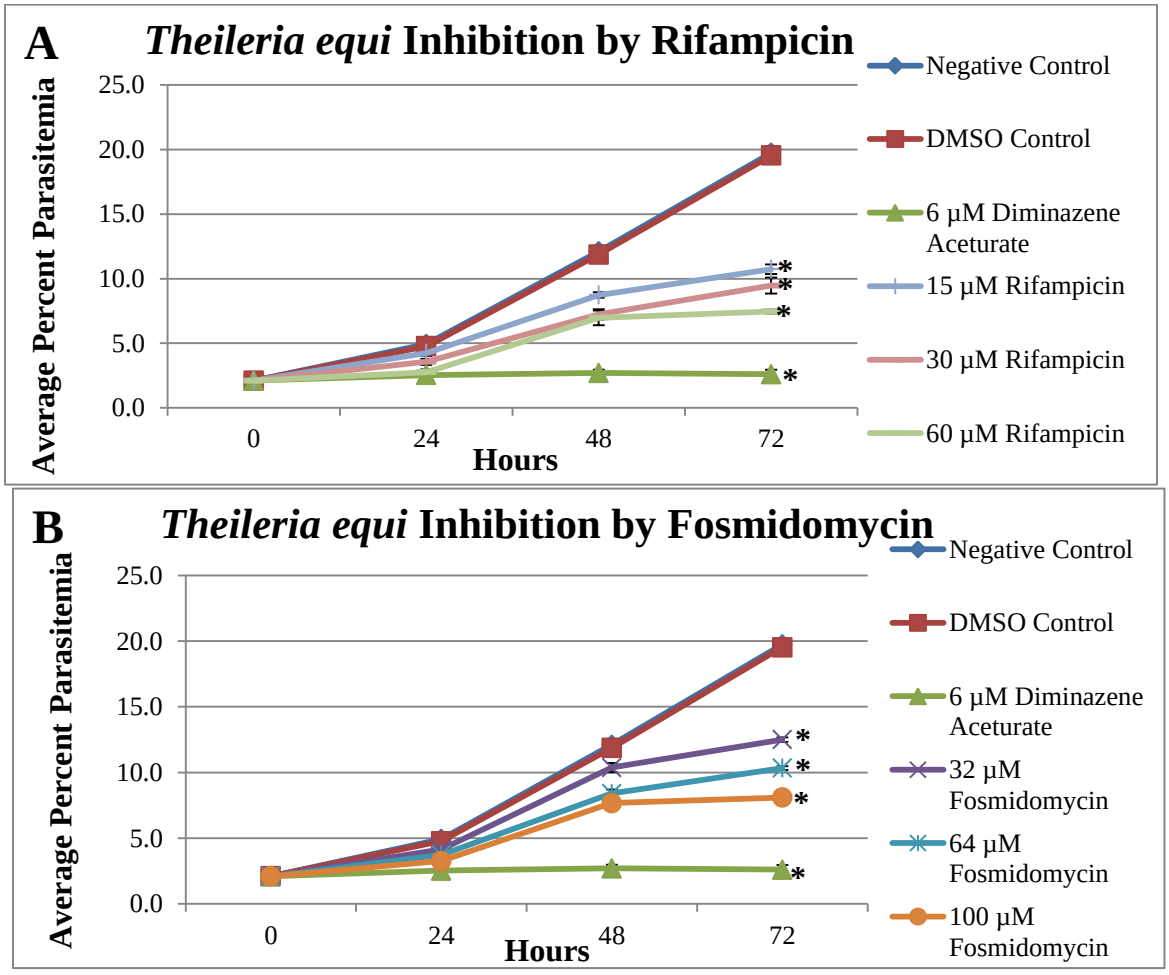

Fig. 3 (A) *In vitro* growth inhibition trials of *T. equi* against a range of concentrations of rifampicin; (B) *In vitro* growth inhibition trials of *T. equi* against a range of concentrations of fosmidomycin. Bars show standard deviations. Points with an asterisk (*) exhibit statistically significant inhibition of growth.

Table 7

Average percent parasitemias of *T. equi* at 72 h treated with rifampicin and fosmidomycin. Cultures treated with complete media, DMSO, and 6.00 µM diminazene aceturate served as controls. *P* values were calculated using a Student's t-test between the 'No Treatment' control and the drug treatment percent parasitemias. *P* values < 0.05 are considered statistically significant.

Treatment	Average Percent Parasitemia	*P* Value
No Treatment	19.7±0.404	N/A
DMSO	19.5±0.252	> 0.500
6.0 µM Diminazene Aceturate	2.6±0.361	< 0.001
32.0 µM Fosmidomycin	12.5±0.173	< 0.001
64.0 µM Fosmidomycin	10.3±0.153	< 0.001
100 µM Fosmidomycin	8.1±0.361	< 0.001
15.0 µM Rifampicin	10.7±0.379	< 0.001
30.0 µM Rifampicin	9.5±0.611	< 0.001
60.0 µM Rifampicin	7.5±0.153	< 0.001

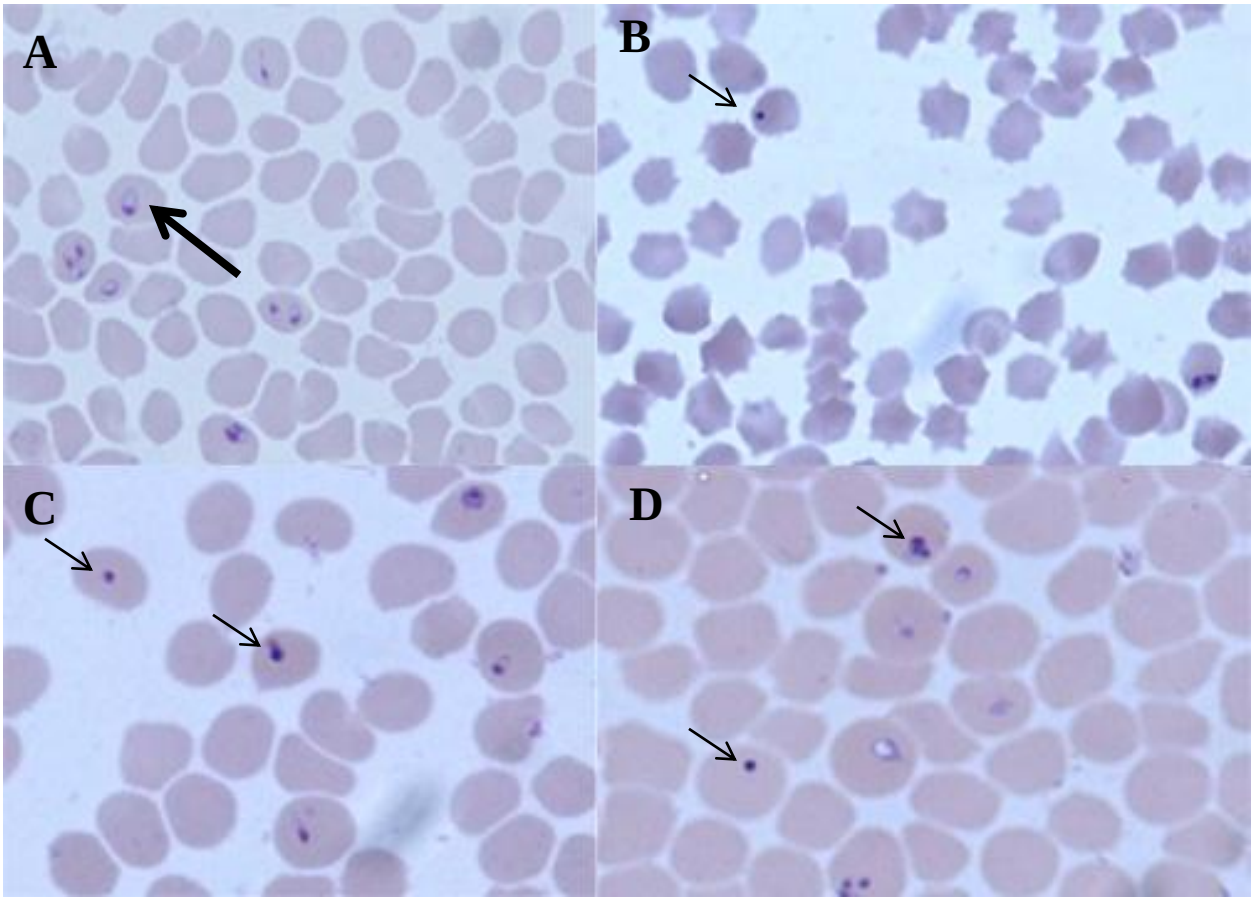

Fig. 4. Giemsa-stained thin erythrocyte smears from 72 h *Theileria equi* cultures under different treatment protocols (1000X). (A) Normal culture, no treatment showing various normal morphological forms; (B) 6.0 µM diminazene aceturate showing the positive control drug effect; (C) 32.0 µM fosmidomycin; and (D) 15.0 µM rifampicin. The larger arrow indicates normal *T. equi* morphology. Short, thin arrows indicate *T. equi* condensed dot forms.

Table 8

Presence of normal-appearing *Theileria equi* parasites at 96 h after no drug treatment for 24 h under different treatment protocols.

Treatment	Presence of Normal-Appearing Parasites at 96 h
Negative Control	+
DMSO	+
6.0 µM Diminazene Aceturate	+
0.75 µM Rifampicin	+
1.5 µM Rifampicin	+
3.0 µM Rifampicin	+
3.75 µM Rifampicin	+
6.0 µM Rifampicin	+
7.5 µM Rifampicin	+
12.0 µM Rifampicin	+
15.0 µM Rifampicin	+
30.0 µM Rifampicin	+
60.0 µM Rifampicin	+
1.5 µM Fosmidomycin	+
3.0 µM Fosmidomycin	+
6.0 µM Fosmidomycin	+
8.0 µM Fosmidomycin	+
12.0 µM Fosmidomycin	+
16.0 µM Fosmidomycin	+
24.0 µM Fosmidomycin	+
32.0 µM Fosmidomycin	+
64.0 µM Fosmidomycin	+
100.0 µM Fosmidomycin	+

Babesia caballi

Initially, rifampicin was tested at 3.75, 7.5, and 15.0 µM against *B. caballi* (Fig. 5A). The negative control and DMSO-treated cultures reached maximum parasitemias of 9.0% and 8.9% respectively at 72 h (Table 9). These parasitemias showed no difference statistically ($P > 0.750$). The positive control cultures, which were treated with 2.4 µM diminazene aceturate, reached a parasitemia of 0.0% at 72 h (Table 9). This was significantly inhibited when compared with the negative control cultures ($P < 0.001$). The cultures treated with 3.75, 7.5, and 15.0 µM rifampicin reached maximum parasitemias of 5.3%, 4.5%, and 4.0% respectively at 72 h (Table 9). Each of these was statistically significantly inhibited in comparison with the negative control cultures ($P < 0.001$, $P < 0.001$, $P < 0.001$, respectively). Fosmidomycin was tested at 8.0, 16.0, and 32.0 µM against *B. caballi* (Fig. 5B) alongside the rifampicin tests in Figure 5A. As such, the control data is the same. The cultures treated with 8.0, 16.0, and 32.0 µM fosmidomycin reached maximum parasitemias of 3.7%, 2.8%, and 2.3% respectively at 72 h (Table 9). Each of these was also inhibited significantly in comparison with the negative control cultures ($P < 0.001$, $P < 0.001$, $P < 0.001$, respectively).

Distinct morphological changes were noticed in the cultures treated with rifampicin, fosmidomycin and diminazene aceturate in comparison with those that were treated with either DMSO or negative control media. The morphology of the parasites under their separate treatment protocols is demonstrated in Figure 7. In the cultures treated with rifampicin, fosmidomycin, or diminazene aceturate condensed dot forms of *B. caballi* predominated (Fig. 6). The IC_{50} for *B. caballi* treated with rifampicin is 6.42. µM. The equation obtained from the graph in Microsoft Excel is shown below.

Equation 3: $Y = 0.0411x^2 - 0.9284x + 8.7494$

The R^2 coefficient obtained via this analysis is equal to 0.9618, suggesting a high degree of correlation.

The IC_{50} for *B. caballi* treated with fosmidomycin is 8.24 µM. The equation obtained from the graph in Microsoft Excel is shown below.

Equation 4: $Y = 0.0131x^2 - 0.14x + 8.6512$

The R^2 coefficient obtained via this analysis is equal to 0.9572 suggesting a high degree of correlation.

Upon removal of drug pressure, parasite growth resumed in all instances for *B. caballi* except for those cultures treated with 2.40 µM diminazene aceturate (Table 10).

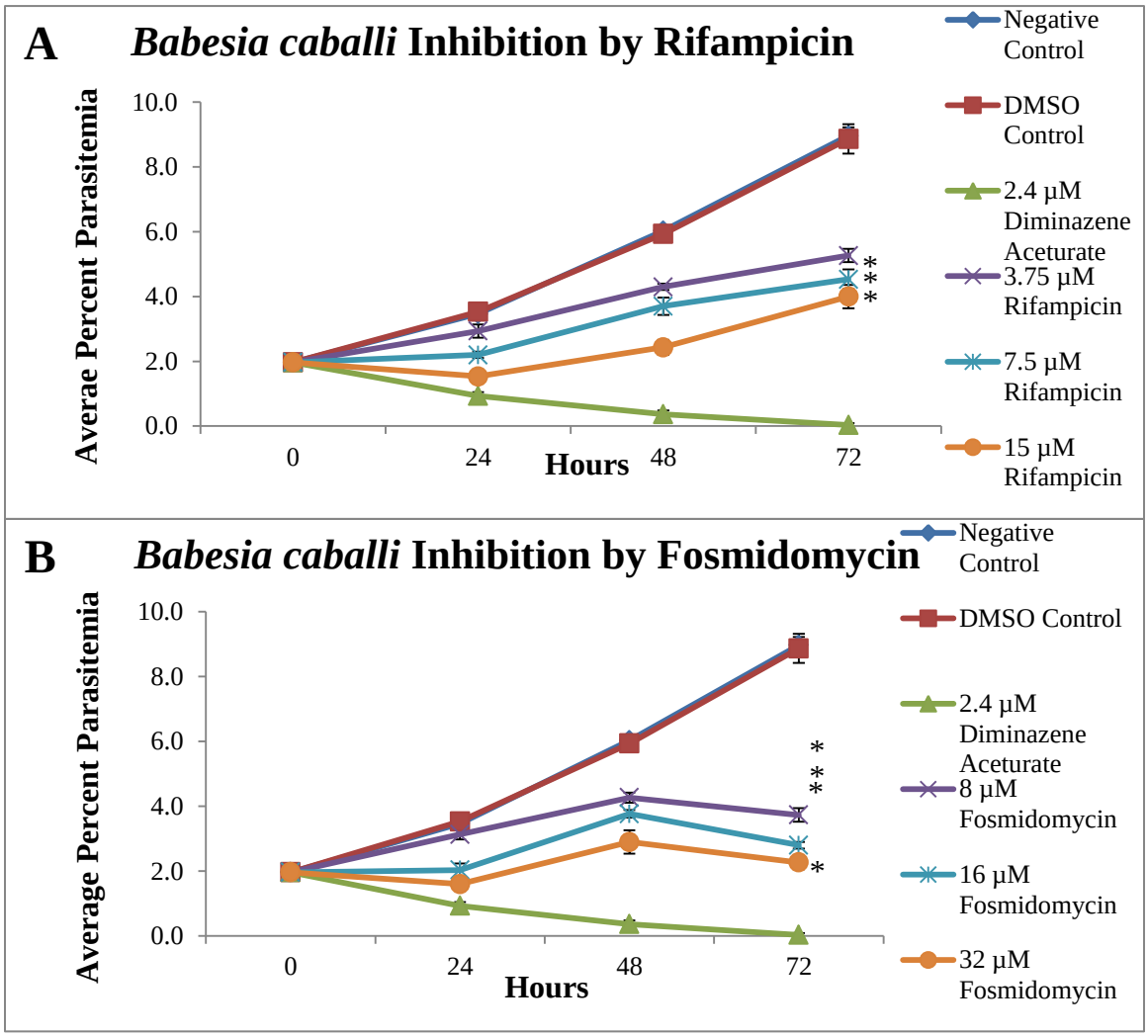

Fig. 5. (A) *In vitro* growth inhibition trials of *Babesia caballi* against a range of concentrations of rifampicin. (B) *In vitro* growth inhibition trials of *B. caballi* against a range of concentrations of fosmidomycin. Bars show standard deviations. Points with an asterisk (*) exhibit statistically significant inhibition of growth.

Table 9

Average percent parasitemias of *Babesia caballi* at 72 h treated with rifampicin and fosmidomycin. Cultures treated with complete media (No Treatment), DMSO, and 2.4 µM diminazene aceturate served as controls. P values were calculated using a Student's t-test between the 'No Treatment' control and the drug treatment percent parasitemias. *P* values < 0.05 are considered statistically significant.

Treatment	**Average Percent Parasitemia**	***P* Value**
No Treatment	8.97±0.252	N/A
DMSO	8.87±0.451	> 0.750
2.40 µM Diminazene Aceturate	0.03±0.058	< 0.001
3.75 µM Rifampicin	5.27±0.208	< 0.001
7.50 µM Rifampicin	4.53±0.306	< 0.001
15.0 µM Rifampicin	4.00±0.361	< 0.001
8.00 µM Fosmidomycin	3.73±0.208	< 0.001
16.0 µM Fosmidomycin	2.80±0.100	< 0.001
32.0 µM Fosmidomycin	2.27±0.208	< 0.001

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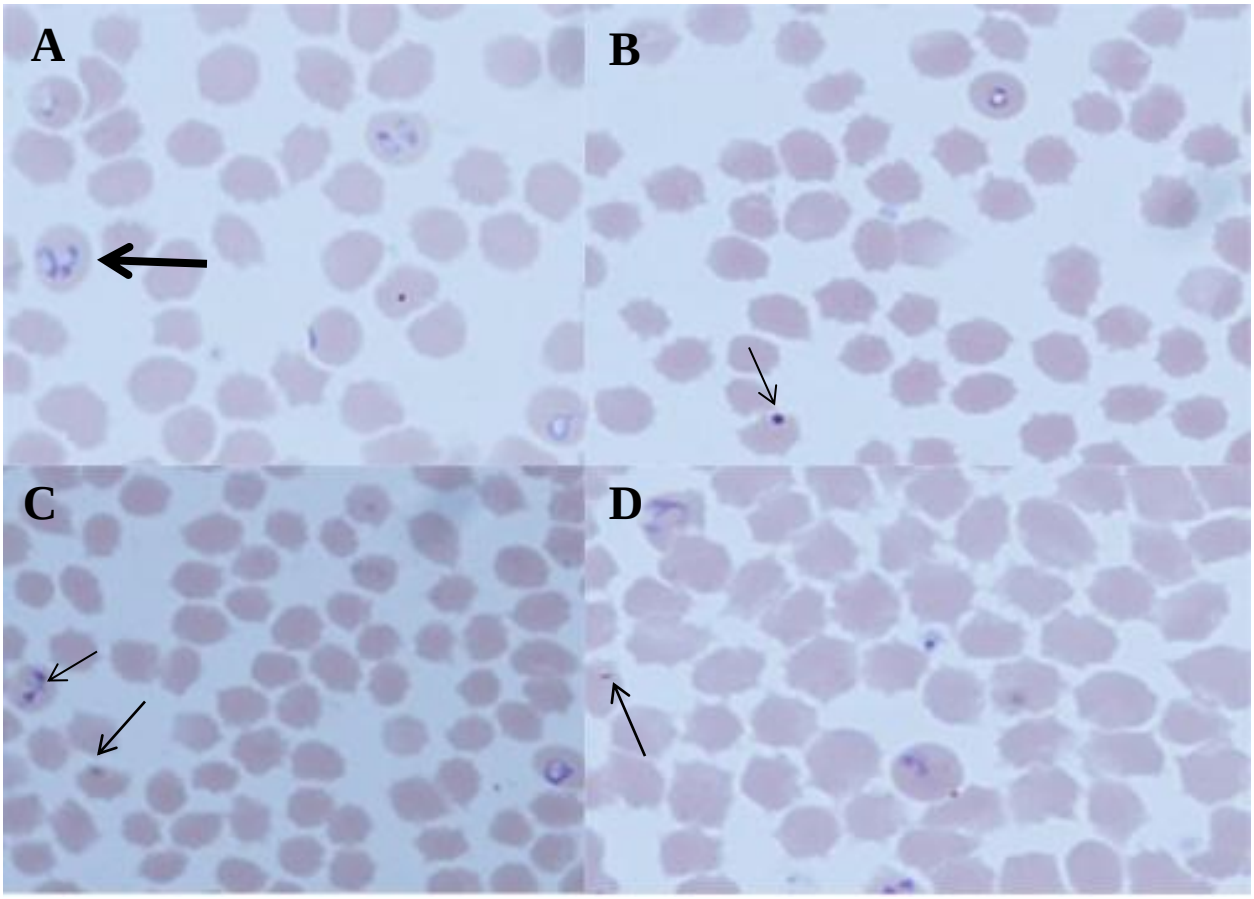

Fig. 6. Giemsa-stained thin erythrocyte smears from 72 h *Babesia caballi* cultures under different treatment protocols (1000X). (A) Normal culture, no treatment showing various normal morphological forms; (B) 2.4 µM diminazene aceturate showing the positive control drug effect; (C) 32.0 µM fosmidomycin; and (D) 15.0 µM rifampicin. The larger arrow indicates normal *B. caballi* morphology. The short, thin arrows indicate *B. caballi* condensed dot forms.

Table 10

Presence of normal-appearing *Babesia caballi* parasites at 96 h after no drug treatment for 24 h under different treatment protocols.

Treatment	Presence of Normal-Appearing Parasites at 96 h
Negative Control	+
DMSO	+
2.40 µM Diminazene Aceturate	-
3.75 µM Rifampicin	+
7.5 µM Rifampicin	+
15 µM Rifampicin	+
8.00 µM Fosmidomycin	+
16.0 µM Fosmidomycin	+
32.0 µM Fosmidomycin	+

CHAPTER IV

CONCLUSIONS

The aim of this study was to determine the anti-protozoal activity of fosmidomycin and rifampicin against the etiological agents of EP. Specifically, it was hypothesized that fosmidomycin would exhibit theilericidal and babesiacidal activity against *Theileria equi* and *Babesia caballi*, respectively while rifampicin would only exhibit static activity of parasite growth. This study successfully demonstrated anti-protozoal activity against the agents of EP, although the hypothesis tested was not proven. The significant inhibition of parasite growth and identification of the IC_{50} for both of these drugs for both parasites clearly indicates that there is *in vitro* inhibition of parasite growth.

Based on this study it cannot be said if fosmidomycin exhibits theilericidal and babesiacidal activity against *T. equi* and *B. caballi*, respectively. Morphological examination of the thin erythrocyte smears from drug-exposed cultures showed what appeared to be a higher proportion of condensed dot forms for both *T. equi* and *B. caballi* with both fosmidomycin and rifampicin. However, our study did not ascertain whether these were viable parasites or not.

Complete clearance of the parasite from the blood continues to be the goal for treatment of EP. This is because even after the signs of disease regress, equids can remain carrier animals, serving as potential reservoirs for future infective outbreaks of EP. For this reason, the trade of EP positive horses is highly restricted. Therefore, there is a clear and pressing need for the identification of treatment that will not only alleviate disease, but clear infection as well. This study was not successful in demonstrating the ability to clear infection with either drug for either parasite under *in vitro* conditions. However, further trials for longer treatment periods and/or with higher drug concentrations are warranted. There is a need to determine if a longer treatment period is associated with complete elimination of EP infection.

Due to the pronounced anti-protozoal activity demonstrated by rifampicin and fosmidomycin alone, studies testing combinations of the drugs should be performed in order to elucidate if synergism exists with these drugs. Also, *in vivo* trials should be done to determine if rifampicin and fosmidomycin could serve as possible drugs for EP treatment. Rifampicin is approved for use in equids whereas fosmidomycin has not been approved (Kohn *et al.*, 1993).

In this study, anti-microbials that had not been tested against EP were found to be effective at depressing parasitemia levels *in vitro*. The positive results of this study indicate a need for examination of other anti-microbials for their efficacy at treating EP. Due to the constant threat of introduction of EP, this research should be considered a priority for equine veterinary researchers in the United States.

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