IN VITRO DRUG SUSCEPTIBILITY OF THEILERIA EQUI AND THE ROLE OF ABC TRANSPORTERS IN RESISTANCE TO IMIDOCARB DIPROPIONATE

By

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To the Faculty of Washington State University:

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This program was supported in large part by Zoetis-Morris Animal Foundation Fellowship Grant No. D12EQ-901.
The primary work presented involves evaluation of the apicomplexan hemoparasite *Theileria equi* for imidocarb dipropionate resistance, assessment of possible alternative treatment options, and investigation of the potential role for ATP-binding cassette transporters in drug resistance. An *in vitro* drug susceptibility assay was developed and adapted to multiple different drugs. The susceptibility of two *T. equi* isolates and a lab generated variant to both imidocarb dipropionate and the experimental bumped kinase inhibitor 1294 was assessed. Two distinct isolates of *T. equi* had an almost four-fold difference in imidocarb dipropionate susceptibility, and a variant generated through continuous *in vitro* imidocarb dipropionate exposure developed a further 15-fold decrease in susceptibility. In contrast, there was no difference in susceptibility to bumped kinase inhibitor 1294, suggesting that these drugs may represent a possible alternative for resistant *T. equi* parasites. Also presented is the failure of four *T. equi* infected ponies to clear infection with the USDA Florida strain despite two rounds of imidocarb dipropionate treatment, a demonstration of drug resistance *in vivo*. 
This collective data raises concern for the existence and emergence of drug resistance at the parasite level. An investigation was undertaken of the 45 putative *T. equi* ATP-binding cassette transporters as potential mediators of imidocarb dipropionate efflux leading to drug resistance. Selection of the most likely candidate transporters based on differential transcription was accomplished using RNA sequencing. No experiments performed in this study supported imidocarb dipropionate as a substrate for efflux by the most differentially expressed ABC transporter BEWA_032300, however there are other transporters of interest and other mechanisms by which ABC transporters can contribute to drug resistance for further investigation.

The described drug assay was utilized in a collaborative project to examine the effect of environmental exposures on the stability and efficacy of buparvaquone and parvaquone. *T. equi* was used as a model organism for *Theileria parva*, for which these drugs are vital in prophylaxis and treatment. It was found that air exposure causes significant drug degradation and subsequent loss of efficacy, a finding that has implications for successful treatment as well as the prevention of drug resistance development.
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DEDICATION

To my husband Devon and my mother Karen,

the two most important and influential people in my life.

I would not be here without your love and support.
GENERAL INTRODUCTION

*Theileria equi* is a tick-borne apicomplexan parasite and an agent of equine piroplasmosis (EP). Although the United States was declared free of EP in 1988, multiple recent U.S. outbreaks emphasize the ineffectiveness of current control programs and the critical need for effective chemotherapeutic strategies for parasite clearance in infected horses. The antiprotozoal drug imidocarb dipropionate has been effective in clearing *T. equi* from the peripheral blood of numerous naturally infected horses. However, treatment has failed in multiple natural and experimental cases.

Evolving drug resistance is commonly observed in other apicomplexans, most notably the human malarial agent *Plasmodium falciparum*, with failure of parasite clearance and recrudescence of infection. Similar resistance to imidocarb dipropionate likely occurs in *T. equi*. *P. falciparum* drug resistance is usually multifactorial; however specific members of the ATP-binding cassette (ABC) family of transporter proteins are known to contribute. Both ABC transporter gene copy number variations as well as single nucleotide polymorphisms have been associated with altered drug susceptibility, with additive effects. The most well-characterized mechanism of transporter-mediated resistance is through drug efflux; however, transporters can contribute to resistance in multiple ways.

Chapter One was published in the journal *Parasites & Vectors* (cited in Chapter Three) and subsequently follows the required format, which was also carried over to Chapter Three. Chapter Two was prepared for submission to the *International Journal for Parasitology: Drugs and Drug Resistance* and therefore represents a different format. Siddra Hines was the sole writer of all manuscripts, although authorship was shared based on research contributions.
CHAPTER ONE

INTRODUCTION

*Theileria equi*, one of the two causative agents of equine piroplasmosis, is a tick-transmitted hemoproteozoan parasite classified within the phylum Apicomplexan. The United States has been considered free of equine piroplasmosis for several decades, however the occurrence of multiple U.S. outbreaks in recent years has caused a resurgence of interest in effective treatment options. Infection can be clinical or subclinical, but persists even with resolution of clinical signs [1-3]. Strict federal regulations for the elimination of infected horses have been established in an attempt to maintain piroplasmosis-free status in the U.S., requiring *T. equi* positive horses to be euthanized, permanently quarantined, exported to the country of origin, or treated under the current USDA-ARS-APHIS treatment program [4,5]. This program is currently the only federally-sanctioned option for treatment in the U.S., as full elimination of parasites from the host must be verified in order for treated horses to no longer be considered potential reservoirs of infection [5].

For most apicomplexan parasitic pathogens, the goal of treatment is to minimize the clinical impact of disease. Complete elimination of this type of pathogen is a considerable challenge, particularly with organisms such as *T. equi* which causes persistent infection [3]. Imidocarb dipropionate (IMD) is a dicationic diamidine of the carbanilide series of antiprotozoal compounds, and is the drug most commonly used to treat equine piroplasmosis caused by both *T. equi* and *Babesia caballi*. In a recent major outbreak localized in Texas [6], IMD successfully cleared over 163 naturally infected horses of *T. equi* (Dr. Angela Pelzel, USDA-APHIS, personal communication)[4,5]. However, variation in response to treatment with an identical IMD
protocol has been observed in both natural and experimental *T. equi* infection [4,7-10], with treatment failure characterized by parasite persistence and recrudescence of parasitemia following discontinuation of treatment. The identification of drug resistance in other apicomplexan parasites [11-13] indicates drug resistance is likely an important factor in *T. equi* treatment failures. In particular, the human malarial agent *Plasmodium falciparum* has exhibited continuously evolving multidrug resistance, necessitating continued development of novel antimalarial drugs for effective treatment. Importantly, failure of treatment with previously effective drug protocols is almost invariably associated with decreased *in vitro* susceptibility to the treatment drug [11,14].

Many drugs have been assessed *in vitro* for efficacy against *T. equi* [15-21]; however a large number of these are not biologically relevant or feasible for use in horses. Although IMD is commonly used clinically, susceptibility has never been evaluated *in vitro* for *T. equi* nor compared between parasite strains. Importantly, the potential impact of IMD exposure on the susceptibility of *T. equi* to this drug, a known factor in the development of drug resistance in many other organisms [14,22-27], has not been investigated.

Given the scarcity of treatment options for *T. equi* and the potential for drug resistance, evaluation of alternative and novel drugs is necessary. Bumped kinase inhibitors (BKIs) are a group of experimental compounds currently being investigated for *in vitro* and *in vivo* efficacy against malaria [28,28,29], toxoplasmosis [30,31], cryptosporidiosis [31,32], and other protozoal diseases [33]. The BKIs selectively inhibit apicomplexan calcium-dependent protein kinases (CDPKs), which are critical for multiple parasitic physiological functions including parasite motility and invasion as well as in secretory pathways and replication [28]. Importantly,
these CDPKs are absent in vertebrates, making them excellent anti-apicomplexan chemotherapeutic candidates [34]. Specifically, BKIs are competitive inhibitors of ATP-binding, and gatekeeper residue size appears to be a major factor in the selectivity of BKIs. These residues in apicomplexan CDPKs are small, typically glycine, serine, or threonine [28,34], which allow access to the ATP-binding pocket for BKIs to bind and inhibit apicomplexan CDPKs. Although CDPKs are not present in mammals, binding of most other mammalian kinases by BKIs is prevented by gatekeeper amino acid residues with large side chains that occlude access to the ATP-binding pocket. Therefore, the BKIs do not inhibit the proliferation of mammalian cells, and have been shown to be non-toxic in rodents [28,29,32].

In the present study, we evaluated the in vitro growth inhibitory effects of IMD against two isolates of T. equi, as well as a variant that was exposed in vitro to the drug. We also describe four ponies infected experimentally that failed to clear T. equi despite two rounds of IMD treatment following the established protocol (4 mg/kg, IM, q72 hrs for four doses) [7]. We then evaluated the in vitro efficacy of a novel bumped kinase inhibitor, BKI compound 1294, against two T. equi isolates with different degrees of susceptibility to IMD. This BKI compound was equally effective against both T. equi isolates, including the variant exposed in vitro to IMD. The results of this work should have implications in the design of therapeutic strategies against infections caused by drug-resistant T. equi.
METHODS

Chemical reagents

For HL2A-FBS and –NHS culture media, HL-1 and HEPES were obtained from Fisher Scientific (Waltham, MA), HB101 supplement from Irvine Scientific (Santa Ana, CA), L-glutamine and AlbuMax from Gibco (Grand Island, NY) and penicillin/streptomycin and gentamicin from Sigma-Aldrich (St. Louis, MO). Hydroethidine was acquired from Invitrogen (Carlsbad, CA) as a 5 mM solution solubilized in DMSO. Imidocarb dipropionate (Imizol®, Merck, Millsboro, DE) at 344 mM (120 mg/mL) was utilized as the stock solution for all IMD assays and diluted in medium to reach experimental concentrations each day for use in the parasite growth inhibition assay described below. Bumped kinase inhibitor compound 1294 was generated as described [35], dissolved in DMSO at 20 mM, and then diluted in medium as above for use in the BKI 1294 growth inhibition assays.

Evaluation of Theileria equi CDPK sequence

Relevant amino acid sequences of BKI-binding CDPK proteins from other apicomplexans [33] including P. falciparum (PfCDPK1 [GI:124801388]), Babesia bovis (BbCDPK4 [GI: 154796736]), and T. gondii (TgCDPK1 [GI:255917998]) were obtained from GenBank and BLASTed (blastp, NCBI) against amino acid sequences predicted in the T. equi genome (GenBank accession number: ACOU00000000) in order to identify the T. equi ortholog. These sequences were then aligned and analyzed using ClustalW and BoxShade programs.

In vitro cultivation of Theileria equi
The USDA Florida strain of *T. equi* (FL) \[36,37\] was obtained as a subculture from ongoing USDA research cultures. Cultures were initially grown in a microaerophilic environment (5% O\(_2\)) in modified HL2A-FBS medium \[38\] with 10 mM hypoxanthine, 200 U/mL penicillin, and 200 μg/mL streptomycin added. Over time, the cultures were adapted to ambient O\(_2\) in a 5% CO\(_2\) 37°C incubator and medium was converted from modified HL2A-FBS to modified HL2A-NHS (substituting normal horse serum for fetal bovine serum). Cultures were maintained in 24 well plates with an erythrocyte concentration of 10% in 1300 μL total well volume and split 1:4 every other day, with 1000 μL of medium changed on intervening days.

A novel *T. equi* isolate (TX) was obtained from a parasitemic horse as a part of a separate previous study \[4\] and adapted into culture. Briefly, an EDTA-anticoagulated whole blood sample was collected and centrifuged at 800 g for 30 minutes. The plasma and leukocytes were discarded, and the erythrocytes washed three times in an equivalent volume of VYM's buffer, centrifuging as before. These infected erythrocytes were then placed in one well of a 24 well plate with 1000 μL of media at a 1:1 ratio with uninfected normal horse erythrocytes (60 μL of each). The plate was initially placed in a 37°C incubator with 5% O\(_2\), 5% CO\(_2\), and 90% N\(_2\) gas, however following successful culture initiation, TX was subsequently adapted to ambient O\(_2\) in a standard 5% CO\(_2\) incubator and maintained as for the FL strain.

**In vitro parasite growth inhibition assay**

Initial IMD concentrations to be used in the assay were determined based on the pharmacokinetics of the drug in horses \[39\] and the *in vitro* susceptibility of other organisms to the drug \[22,40,41\]. To evaluate the dynamics of parasite growth during IMD exposure and to
determine the appropriate incubation time for the assay, the FL strain was cultured in a 24-well plate for 72 hours with assessment of parasite growth via flow cytometry (below) and one mL medium change every 24 hours. Serial dilutions of IMD were tested in triplicate ranging from 1 nM to 775 nM, with a target starting percent parasitized erythrocytes (PPE) for all wells of 0.25-0.5%.

Based on these results, it was determined that the assay should be performed over 72 hours, starting at a low initial PPE. Further assays were performed in a 96-well plate, with a final erythrocyte concentration of 9% and target starting PPE of approximately 0.3%. Two-fold serial dilutions of IMD ranged from 2.7 nM to 344 nM, and triplicate samples for each concentration, including infected untreated controls, were evaluated. Triplicate wells of normal uninfected erythrocytes were also included as a control comparison. For assay initiation, 2x drug concentrations were prepared, with 100 µL of the appropriate concentration placed into each well with 100 µL of normal medium and 20 µL of RBCs to reach the final 1x drug concentration for the assay and approximately 9% RBC concentration in each well. At 24 and 48 hours, 160 µL of medium containing the appropriate 1x drug concentration was changed in each well, with plain medium used for controls. The assay was then evaluated at 72 hours. Complete growth inhibition was considered to be achieved when the PPE remained at the starting level of 0.3-0.5% after 72 hours, indicating no active parasite growth.

The chemical structure of BKI 1294 has been previously published [30]. Drug concentrations tested for BKI 1294 included 0.3 µM, 3 µM, 6 µM, 9 µM, 12 µM, and 30 µM. Assay procedure was otherwise identical to that for IMD.
Flow cytometric evaluation of parasite growth

After 72 hours, all samples were transferred to a 96 well V-bottom plate to be stained with hydroethidine. Cells were centrifuged at 500 g at room temperature, and the culture medium supernatant discarded. They were then washed in 150 μL of 1X PBS per well and centrifuged as before. Stock 5mM hydroethidine in anhydrous DMSO was diluted to 20 μg/mL in 1X PBS and added at to all samples (including normal erythrocytes) at 100 μL per well. Samples were incubated for 15 minutes at 37°C in 5% CO₂ in the absence of light, and 100 μL of plain 1X PBS was added at the conclusion of the incubation prior to centrifugation. After removal of the supernatant, the cells were resuspended in 200 μL of 1X PBS. Fifty μL of each sample was then diluted into approximately two mL of 1X PBS containing 0.2% sodium azide for flow cytometric analysis.

Cell suspensions were evaluated using a FACSCaliber flow cytometer equipped with CellQuest computer software (Becton Dickinson Immunocytometry Systems, San Jose, CA) on a Macintosh computer. The inclusion gate was based on the forward and side scatter features of uninfected erythrocytes stained with hydroethidine, and 50,000-150,000 events per sample were collected. Ethidium bromide fluoresces in the FL-2 channel, therefore argon-laser fluorescence excitation at 488 nm and emission at 585 nm (range 563-607 nm) were used for analysis in log Fl 2 data mode. Fluorescent profiles were recorded for later analysis with FCS Express software (De Novo software, Los Angeles, CA). Quadrant gating of generated dot plots (Fl-2 vs. side scatter) was based on stained uninfected erythrocyte controls to delineate between infected and uninfected cell populations.
**Determination of IC\(_{50}\) and IC\(_{90}\) values**

The PPE of each well was determined based on the percentage of the cell population characterized as RBCs that exhibited FL-2 fluorescence. Mean PPE for each drug concentration and controls was calculated by averaging the PPE of all three triplicate wells. Individual PPE values that deviated from the other replicates by >0.5% were considered outliers and eliminated from data evaluation. Percent of maximum PPE was then calculated for each concentration using the mean PPE value in comparison to the highest PPE obtained in the assay for a given isolate. The 50% (IC\(_{50}\)) and 90% (IC\(_{90}\)) inhibitory concentrations were determined by fitting the curves with nonlinear regression using GraphPad Prism version 6.03 for Windows (GraphPad Software, La Jolla, CA).

**Exposure of *Theileria equi* parasites to imidocarb dipropionate in vitro**

Exposure of the FL strain of *T. equi* to IMD was performed via two different methods. The first method involved pulse exposure, with parasites exposed at 28.7 nM (the approximate IC\(_{50}\) value determined previously) for an initial period of 11 days, until growth had declined to a PPE of less than 1.5%. Drug pressure was then removed and the parasites were allowed to recover for five days. They were then re-exposed to 28.7 nM for 24 hours. Subsequent 24 hour re-exposures of the pulse exposure variant to increasing concentrations of IMD were performed over the next two months, allowing sufficient time for recovery between exposures, with the final re-exposure at 172 nM (FL Exp variant 1). The third method of FL IMD exposure involved continuous exposure to increasing concentrations of IMD, starting at the lowest
previously determined IC₅₀ of the strain (24 nM) and gradually increasing up to 115 nM (FL Exp
variant 2).

The TX isolate was exposed continuously to 3.2 nM IMD for 24 days and also to a higher
concentration (9.5 nM IMD) for a short period of time (11 days).

**Infection and treatment of ponies with *Theileria equi***

Four naïve mixed-breed ponies, 1-2 years of age, were experimentally infected with the
USDA FL strain of *T. equi* via *Rhipicephalus microplus* tick transmission as a part of a separate
research study, using previously published methods [42]. All ponies were confirmed positive for
*T. equi* by nested PCR [4] and were initially treated with IMD (4 mg/kg, IM, q72 hrs for four
doses) at seven months post-infection. Post-treatment infection status was determined using
nested PCR and cELISA [43]. Because this initial treatment failed to clear *T. equi* in each of the
four ponies, the same treatment regimen was repeated 18 months post-infection and the same
follow-up evaluations performed. All experiments involving animals were carried out in
accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals
of the National Institutes of Health and in conformance with the United States Department of
Agriculture animal research guidelines, under a protocol approved by the Washington State
University Institutional Animal Care and Use Committee (ASAF # 04163).
RESULTS

Imidocarb dipropionate treatment of ponies experimentally infected with the USDA FL strain of *Theileria equi*

Sixteen days following the first round of IMD treatment (seven months post-infection), all four ponies appeared to be negative for *T. equi* based on nested PCR (Figure 1a). However, nested PCR was repeated at 17 months post-infection and all ponies were found to be positive. After the second round of IMD treatment at 18 months post-infection, the ponies again appeared negative for *T. equi* based on nested PCR performed 16 days post-treatment. However, re-evaluation 37 and 80 days (Figure 1b) after this second round of treatment (19-20 months post-infection) confirmed that all ponies again reverted to a positive state and that two rounds of IMD treatment failed to clear infection. Seropositivity was also confirmed at 14 and 20 months post-infection by cELISA (data not shown).

In vitro susceptibility of *Theileria equi* to imidocarb propionate

Development of the *in vitro* parasite growth inhibition assay using IMD for *T. equi* was successfully accomplished using the FL parasite strain, with evaluation of the PPE for each sample using flow cytometry (Figure 2). Regardless of the concentration of IMD, the PPE in all wells doubled or tripled over the first 24 hours. By 48 hours, the growth dynamics across IMD concentrations began to diverge, with parasite growth at higher concentrations leveling off and that at lower concentrations continuing to increase until reaching a maximum of 7-8% PPE at 72 hours. Overall, the final PPE across the range of drug concentrations reflected a dose-dependent effect of IMD, with an IC$_{50}$ of 24 nM (Figure 2a). An IC$_{90}$ could not be determined for
this strain, because even at the maximum drug concentration (775 nM) the PPE still reached 1.2%, equivalent to 15.4% of the growth of untreated parasites. This indicated that the FL strain parasite population more than doubled from the starting PPE of 0.5% even at high IMD concentrations, reflecting a lack of complete growth inhibition.

The TX isolate originating from the 2009 outbreak [4,6] was consistently more susceptible to IMD than the FL strain, with an IC₅₀ of 6.4 nM; almost six-fold lower than FL (Figure 3a). In addition, an IC₉₀ of 26 nM could be determined for the TX isolate, as it did not actively grow at higher drug concentrations, with PPE remaining at the starting level of 0.5%.

**Imidocarb dipropionate exposure of *Theileria equi* and the effect on IC₅₀**

Continuous exposure of the FL strain to increasing concentrations of IMD (up to 115 nM) increased the IC₅₀ a maximum of 15-fold in FL Exp variant 2 to 414 nM (Figure 3b). Pulse exposure with IMD (up to 172 nM) increased the IC₅₀ in FL Exp variant 1 to a lesser degree, approaching four-fold at 94 nM (Figure 3b). However, this effect could not be duplicated in the TX isolate. Despite repeated attempts to expose TX to IMD, the IC₅₀ remained the same at approximately 6 nM. Exposure of TX to a higher concentration of IMD (9.5 nM) resulted in irrevocable decline in culture PPE and eventual death of all exposed parasites.

**Gatekeeper residue of *Theileria equi* calcium-dependent protein kinase**

Due to the variability in response to IMD, alternative drugs for treatment of *T. equi* were considered. The BKI compounds were of interest, and therefore evaluation of the *T. equi* transcriptome for an appropriate CDPK drug target was undertaken. Amino acid sequence
Comparisons of the BKI-binding CDPKs in *P. falciparum*, *B. bovis*, and *T. gondii* against the *T. equi* predicted transcriptome identified the same protein kinase domain-containing protein (GI: 510911326) as the putative CDPK ortholog in *T. equi*. Alignment of these four sequences revealed a threonine gatekeeper residue in *T. equi*, consistent with the small amino acid gatekeeper residues present in comparison organisms (Figure 4), all of which are susceptible to BKI compounds [33].

**In vitro susceptibility of *Theileria equi* to the bumped kinase inhibitor compound 1294**

For BKI 1294, the IC$_{50}$ of the FL Exp variant 1 and the TX isolate were similar (4.212 μM and 3.887 μM respectively) (Figure 5). Active parasite growth of both isolates was completely inhibited at the highest drug concentrations, with PPE limited to the starting level of 0.3% (3.5-3.6% of the maximum PPE). Therefore, the IC$_{90}$ was achieved for BKI 1294 for both FL Exp variant 1 and TX (11.502 μM and 10.964 μM, respectively).
DISCUSSION

Although the life cycle of *T. equi* in horses is biphasic, with an early pre-erythrocytic schizogony within leukocytes, this schizont stage is transient and does not appear to play a role in persistent infection of horses [42]. Instead, long term infection is perpetuated through infection and proliferation of merozoites in erythrocytes, making this merozoite stage most relevant for chemotherapeutic intervention. Therefore, erythrocyte cultures of merozoites were used in this study to evaluate *in vitro* susceptibility. The observed difference in IMD susceptibility between isolates, with the IMD IC₅₀ of the FL strain almost six-fold higher than that of the TX isolate, was consistent with variation in efficacy that has been observed clinically. The overall success rate of IMD treatment for horses infected with the TX isolate during the 2009 outbreak was extremely high [4]. In contrast, the USDA FL strain has been used in multiple experimental settings to evaluate the efficacy of IMD *in vivo*, with infected horses often failing to clear FL following treatment with a protocol identical to that used in the TX outbreak [7,8]. This situation was also observed in the current study, with four FL-infected ponies failing to clear infection despite two rounds of IMD treatment at 4 mg/kg every 72 hours for 4 doses per round. Interestingly, the ponies appeared negative immediately after treatment, with parasite growth suppressed by IMD to the point that it could not initially be detected even with the extremely sensitive nested PCR method, which has been shown to detect a level of parasitemia of 0.000006% [44].

Previous research has demonstrated the presence of *T. equi* in the spleen of asymptomatic horses at times where it was undetectable in the peripheral blood via multiplex PCR [2]. Additionally, it is possible that the level of parasitemia was simply lower than the detectable
limit, and that the small number of parasites surviving drug treatment was still sufficient to allow continued infection after a period of parasite recovery. The lack of complete *in vitro* inhibition of FL is consistent with the observed failure of IMD to clear the FL strain in the infected ponies described in this study, as incomplete parasite inhibition led to a resurgence of parasitemia *in vivo*. This also raises further concern about the potential for development of resistance in these IMD-exposed surviving parasites.

The pharmacokinetics of IMD administered intramuscularly in horses demonstrates a maximum plasma concentration of 0.2 µg/mL (574 nM) at a dose of 2.4 mg/mL [39]. Although this concentration greatly exceeds the IC₅₀ of 24 nM observed for the FL strain in the present study, this plasma concentration is maintained for only 2 hours *in vivo* and drops to undetectable levels (less than 0.0125 µg/mL, or 36 nM) after 12 hours [39]. In contrast, the parasites in the present *in vitro* assay were exposed to IMD continuously at the tested concentrations for 72 hours, and in the case of FL, actively grew despite exposure to high concentrations of drug.

It has been postulated that a reservoir effect exists for IMD *in vivo*, as the drug is deposited in the liver and kidneys [45-48] as well as muscle in cattle [48] during the initial distribution phase. Trace amounts have been detected in the plasma of sheep up to four weeks after treatment [47]. This likely accounts for the overall efficacy of IMD for certain hemoproteozoan parasites, but makes it difficult to compare *in vitro* IC₅₀ results with circulating IMD concentrations to determine if adequate drug concentrations are reached in the plasma. Additionally, the minimum detectable concentration of 0.0125 µg/mL (36 nM) for the HPLC assay utilized in the previous study [39] exceeded the IC₅₀ of IMD for both FL and TX observed in
the current study. Therefore, it is possible that the plasma concentration remains higher than the IC$_{50}$ for a period longer than that suggested by the previous pharmacokinetics study. The 2.4 mg/kg dose used in that study was less than the 4 mg/kg dose currently recommended for clearance of *T. equi* [4,7]. Although the higher dose would be expected to achieve higher plasma concentrations, the fact that treatment failures occur at the higher dose indicates that IMD plasma concentrations above the IC$_{50}$ are not always adequate for elimination of *T. equi* *in vivo*.

We observed that the IC$_{50}$ for the FL strain could be further increased *in vitro* by exposure to IMD, as has been shown previously *in vitro* for *B. bovis* [22]. This finding suggests that resistance could emerge in natural parasite populations with exposure to IMD when animals are treated, particularly if complete inhibition of parasite growth cannot be achieved. As previously mentioned, trace amounts of IMD can be found in the plasma of sheep up to four weeks following treatment with IMD [47], which could result in prolonged drug selection pressure if the same occurs in horses. Although horses are not currently treated routinely in the United States, treatment occurs much more frequently in endemic countries. In these endemic countries the goal of therapy is to treat clinical disease rather than eliminate the parasite entirely from the host [49]. Therefore, treated horses can remain chronically infected and retain a population of parasites that was exposed to IMD yet was not killed by the drug. The possibility of developing IMD resistance is concerning, as there are very few options currently available for treatment of *T. equi*. In contrast to the observations with the FL strain however, IMD susceptibility in the TX isolate was not altered despite repeated IMD exposure. Thus, the capacity for drug exposure-associated changes in *in vitro* IMD susceptibility varies among *T.
equi strains. The underlying mechanism of IMD resistance among different T. equi strains is a focus of ongoing investigation.

The BKIs represent a novel class of compounds with potential as safe and effective treatment alternatives for T. equi infection, particularly for horses infected with a strain that is less susceptible to IMD. Compound 1294 is safe in vivo in rodents and effective against T. gondii [30], C. parvum [32], P. falciparum [29] and B. bovis [33]. Importantly, it is nontoxic in mice following twice daily oral administration of 100 mg/kg for five days [29]. It has 91% oral bioavailability in rats and is likely cleared by hepatic metabolism [29]. In the current study, this compound demonstrated equal and substantial efficacy in vitro against both the TX isolate and the FL Exp variant 1, which had an almost 15-fold difference in IMD susceptibility. Importantly, BKI 1294 resulted in complete parasite growth inhibition for both isolates, consistent with its novel mechanism of action. Although the IC_{50} of BKI 1294 was higher than that of IMD in vitro, its apicomplexan-selective target and its favorable pharmacokinetics and safety in other mammals [28-30] suggest that it could be useful for eliminating T. equi infection in horses. Further investigation of this class of compounds for this purpose is a focus of ongoing research.

Flow cytometry has been used to evaluate parasite infection of erythrocytes for multiple hemoprotozoan species, including B. bovis [50], Anaplasma marginale [51], B. gibsoni [52], B. canis [53], and P. berghei [54]. This technique relies on staining of infected erythrocyte populations with hydroethidine, which is taken up by live parasites within intact erythrocytes and converted to the fluorochrome ethidium bromide. Ethidium bromide intercalates into the DNA of viable parasites and can be utilized to differentiate infected erythrocytes from those that are uninfected or that contain non-viable parasites using flow cytometry [50]. It is
therefore particularly relevant for assessment of drug susceptibility as only viable parasites are identified, in comparison to the traditional method of evaluating PPE by blood smear in which parasite viability cannot be determined. In this study, we were able to successfully apply this technique for *T. equi*-infected erythrocytes for the first time to evaluate parasite growth in drug susceptibility assays.
CONCLUSIONS

This study demonstrated variation in IMD susceptibility for the first time in vitro between two *T. equi* strains, with decreased susceptibility and incomplete parasite inhibition for the USDA FL strain. This finding was consistent with the inability of IMD to clear the same strain in vivo in four experimentally infected ponies, despite two rounds of treatment. Importantly, the emergence of IMD resistance during exposure to the drug was demonstrated in vitro. In contrast, no such in vitro variation in susceptibility between strains was observed for BKI compound 1294. This novel class of compounds may represent an effective alternative for the treatment of resistant *T. equi* infections in horses.
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Figure 1: Nested PCR detection of *T. equi* infection in ponies after failed IMD treatment

(a) 2% agarose gel showing results from packed erythrocytes collected 16 days after the second round of IMD treatment. Lane 2: negative control erythrocytes from an uninfected horse (-). Lanes 3, 4, 5, 6: erythrocytes from the four IMD treated ponies. Lane 7: positive control erythrocytes from a known infected horse showing the 200 bp *T. equi* *ema*-1 amplicon (+).

(b) *T. equi* *ema*-1 amplicons from packed erythrocytes collected 80 days after the second round of IMD treatment. Lanes 2, 3, 4, 5: erythrocytes from the four IMD treated ponies. Lane 6: negative control (-). Lane 7: positive control (+).
Figure 2: Flow cytometric scatter plots depicting percent parasitized erythrocytes (PPE)
Representative samples from a 72 hour growth inhibition assay. Parasitized erythrocytes stained with hydroethidine were detected in the FL-2 channel, and are represented in the bottom right quadrant.
(a) Normal uninfected erythrocytes stained with hydroethidine (negative control).
(b) *T. equi*-infected erythrocytes incubated without imidocarb dipropionate (IMD; positive control).
(c) *T. equi*-infected erythrocytes incubated with 2.76 μM IMD.
Figure 3: Variable *in vitro* susceptibility to IMD among different *T. equi* isolates

Each point on the nonlinear regression curve represents the mean percent of the maximum PPE determined in triplicate wells. IC$_{50}$ is drug concentration resulting in 50% of the maximum PPE detected in wells not containing drug.

(a) Susceptibility of the USDA FL strain (red) and the TX isolate (purple).

(b) Susceptibility of FL Exp variant 1 (purple) and FL Exp variant 2 (green).
Figure 4: Amino acid alignment through the ATP-binding domains of calcium dependent protein kinases

Includes amino acid sequences from *P. falciparum* (Pf), *T. equi* (Te), *B. bovis* (Bb), and *T. gondii* (Tg). The ATP-binding region is boxed, and the gatekeeper residues are shaded.
Figure 5: *In vitro* susceptibility of *T. equi* isolates to BKI compound 1294

Each point on the nonlinear regression curve represents the mean percent of the maximum PPE determined in triplicate wells. IC\(_{50}\) is drug concentration resulting in 50% of the maximum PPE detected in wells not containing drug. Depicted results represent TX (purple) and FL Exp variant 1 (red).
CHAPTER TWO

INTRODUCTION

Buparvaquone (BPQ) and parvaquone (PQ) are hydroxynaphthoquinone compounds that are used to prevent and treat theilerial diseases such as *Theileria parva* (Muraguri et al., 1999) and *Theileria annulata* (Hashemi-Fesharki, 1991). *T. parva* is the causative agent of East Coast Fever (ECF) in cattle, which is a disease of serious clinical and economic importance in eastern and southern Africa. Historically it has been estimated that over a million cattle may be lost annually to ECF in this region (Mukhebi et al., 1992). The fatality rate is approximately 50-60% in zebu cattle (*Bos indicus*), but approaches 100% in European cattle breeds (*Bos taurus*) (Muraguri et al., 1999; Muraguri et al., 2006). *T. parva* is lymphodestructive in nature, causing dramatic immunosuppression (Dolan et al., 1988). Damage to mucous membranes commonly causes serious pulmonary edema, and affected cattle have a profoundly increased risk of developing secondary respiratory and gastrointestinal infections requiring additional treatment. Therefore, early and effective medical intervention is necessary for disease control on an individual and herd level. Prompt treatment with either BPQ or PQ can achieve success rates of 90-100% in uncomplicated cases of ECF (Muraguri et al., 1999). This approach is more cost effective and efficacious than the alternative and expensive process of controlling the brown ear tick (*Rhipicephalus appendiculatus*), the primary vector of this disease (Mukhebi et al., 1992).

In addition to *T. parva*, both BPQ and PQ have been evaluated *in vivo* and found to be somewhat efficacious for the treatment of *Theileria equi*, a causative agent of equine piroplasmosis (Kumar et al., 2003; Kuttler et al., 1987; Salib et al., 2012; Zaugg and Lane, 1989;
Zaugg and Lane, 1992). In the United States where equine piroplasmosis has been eradicated since 1988, the only acceptable treatment outcome is complete clearance of the pathogen from infected horses. Therefore, hydroxynaphthoquinone drugs have not been considered suitable as an alternative independent treatment option.

Veterinarians in East Africa are often called upon to treat ECF in less than ideal conditions. Drugs can be exposed to extreme temperatures (both low and high), extended exposure to ambient air, and potentially exposure to ultraviolet (UV) light if not stored properly; all conditions that are outside the standard recommendations of drug manufacturers. This can be exacerbated in communities where farmers manage disease treatment and prevention (i.e. Maasi communities) in the absence of controlled storage conditions. Although these drugs are manufactured in airtight vials for injection, multi-dose vials invariably allow introduction of atmospheric air, particularly when the bottles experience frequent swings from hot to cold temperatures. It is also possible for practitioners to inadvertently or intentionally inject air when pulling drugs from such vials.

This has been an issue of importance in human medicine, with concern for the stability of antimalarials and other drugs vital for human health in sub-Saharan Africa. Many medications do not reach their labeled shelf life with adequate drug content as a result of extreme environmental conditions (Amin and Kokwaro, 2007; Ballereau et al., 1997; Kayumba et al., 2004). Exposures to UV light, elevated temperature, and atmospheric air are all known factors that can affect drug stability, although the degree to which a drug is affected varies (Amin and Kokwaro, 2007; Ammann, 2011; Chatzitakis et al., 2008; Jaffe et al., 1976; Kayumba et al., 2004; Langner and Maibach, 2009; Risha et al., 2002; Risha et al., 2003; Twagirumukiza et
al., 2009). We investigated the effect of each of these factors on the stability and effectiveness of both PQ and BPQ using a combination of high performance liquid chromatography (HPLC) and a novel *in vitro* parasite system based on *T. equi* as a model organism.
MATERIALS AND METHODS

Chemical reagents

For the primary exposure experiment, chemical grade parvaquone was diluted in dimethyl sulfoxide (DMSO) to 15 mg/mL as the clinical product was not available at the time. For later replication of the air exposure experiment, we successfully obtained the 150 mg/mL clinical product Parvexon (Biomeda, Dublin, Ireland). In both experiments, the clinical buparvaquone product Bupaquone (Eagle Vet. Tech Co., Ltd, Chungnam, Korea) was used. For the initial experiment BPQ was diluted to 5 mg/kg in DMSO, and in the follow-up experiment both buparvaquone and parvaquone were diluted in N-methyl-2-pyrrolidone to their respective concentrations of 5 mg/kg and 15 mg/kg.

Reagents for *T. equi* culture medium (HL2A-NHS) included HL-1 and HEPES (Fisher Scientific, Waltham, MA), HB101 supplement (Irvine Scientific, Santa Ana, CA), L-glutamine and AlbuMax (Gibco, Grand Island, NY) and penicillin/streptomycin and gentamicin (Sigma-Aldrich, St. Louis, MO). Hydroethidine for flow cytometric evaluation was acquired from Invitrogen (Carlsbad, CA) as a 5 mM solution solubilized in DMSO.

Drug exposure conditions

A 1 mL aliquot of each drug (5 mg/mL BPQ and 15 mg/mL PQ, diluted in DMSO) was placed into 1.5 mL microcentrifuge tubes for each sample. A total of 12 samples were utilized for each storage condition (Ambient air, UV light, 37°C, and Combination), with triplicate samples for each designated length of exposure. All samples were wrapped in aluminum foil.
and stored at room temperature, unless under active exposure to UV light or when stored at 37°C.

Tubes containing samples for air exposure had 2 mm holes punched through each cap to simulate puncture by a large gauge (14-16 gauge) needle such as those typically used in large animal medicine. Three samples were removed and stored in fully sealed microcentrifuge tubes at seven (Air7d), 14 (Air14d), 30 (Air30d), and 50 days (Air50d). Exposure to UV light was accomplished using a BSL-II hood UV lamp (UV-A and UV-B). Three samples were pulled after three (UV3h), six (UV6h), 12 (UV12h), and 24 h (UV24h). Outdoor environmental temperature exposure was simulated by placing samples in a 37°C incubator for eight hours per exposure. Three samples were removed after five (37°C5exp), 10 (37°C10exp), 20 (37°C20exp), and 40 exposures (37°C40exp). Between exposures, samples were stored at 4°C in the dark. Finally, for combination exposure each condition was set up as described above. Three samples were exposed to each of the following combinations of conditions: 1) Five exposures 37°C/three hours UV light/seven days ambient air (Combo7d), 2) 10 exposures 37°C/six hours UV light/14 days ambient air (Combo14d), 3) 20 exposures 37°C/12 hours UV light/30 days ambient air (Combo30d), 4) 40 exposures 37°C/24 hours UV light/50 days ambient air (Combo50d).

**BPQ/PQ assay development and determination of drug susceptibility of exposed samples**

Ongoing laboratory cultures of *T. equi* infected erythrocytes were utilized for this assay. These parasites were isolated and adapted into culture from a splenectomized horse infected with *T. equi* originating from a 2009 field outbreak in Texas (Hines et al., 2015; Ueti et al., 2012). Fresh PQ and BPQ were both evaluated for efficacy in this system. The initial concentrations
tested were based on described pharmacokinetics of PQ and BPQ in cattle (Kinabo and Bogan, 1988; Muraguri et al., 2006), in vitro efficacy for T. parva in lymphoblastoid culture (McHardy et al., 1985), and in vitro efficacy for Leishmania donovani (Croft et al., 1992; Venkatesh et al., 2008), with two-fold dilutions initially ranging from 0.00019 to 0.048 µg/mL for PQ and 0.0003 to 0.0768 µg/mL for BPQ. These dilutions were prepared using the stock solutions in DMSO of PQ (15 mg/mL) and BPQ (5 mg/mL), serially diluted in HL2A-NHS media to reach the appropriate concentrations for the assay. The final concentration of DMSO in all drug dilutions was < 0.02%, which has been demonstrated to have no effect on parasite growth (Wise et al., 2012).

The assay was adapted from the procedure described by Hines 2015. Briefly, each assay was performed over a 48 hour period in a 96-well plate, with infected/untreated erythrocytes, uninfected/untreated erythrocytes, and infected/treated erythrocytes (at each tested concentration of drug) used as controls. Each sample and control was evaluated in triplicate, with the target starting percent parasitized erythrocytes (PPE) in each well at approximately 1.0%. Hydroethidine staining with flow cytometric analysis was used to evaluate the final PPE for each sample. (Hines et al., 2015).

Each of the three independent exposure replicates for every exposure type and length was assessed in tandem using three technical replicates for each drug concentration. The concentrations initially tested for PQ included two-fold dilutions ranging from 0.00075 to 0.048 µg/mL, and from 0.0006 to 0.0096 µg/mL for BPQ. This range was expanded upwards as necessary to accommodate samples with an increase in IC50. Evaluation of exposed samples began with examination of maximum Combo exposures (BPQ Combo50d and PQ Combo50d) to
determine the expected maximum possible effect from all factors, and then experiments with other exposures followed. A similar process was used for individual conditions, starting with UV$_{24h}$, 37°C$_{40exp}$, and Air$_{50d}$. If no treatment effects were evident upon maximum exposure, lesser exposures were not evaluated.

**Flow cytometric analysis**

Cell suspensions were evaluated using a FACSCaliber flow cytometer equipped with CellQuest computer software (Becton Dickinson Immunocytometry Systems, San Jose, CA) on a Macintosh computer. The inclusion gate was based on the forward and side scatter features of uninfected erythrocytes stained with hydroethidinium and 50,000-150,000 events per sample were collected. Ethidium bromide fluoresces in the FL-2 channel and subsequently argon-laser fluorescence excitation at 488 nm and emission at 585 nm (range 563-607 nm) were used for analysis in log Fl 2 data mode. Fluorescent profiles were recorded for later analysis with FCS Express software (De Novo software, Los Angeles, CA). Quadrant gating of generated dot plots (Fl-2 vs. side scatter) was based on stained uninfected erythrocyte controls to delineate between infected and uninfected cell populations. The PPE of each well was determined based on the percentage of the cell population characterized as RBCs that exhibited FL-2 fluorescence on flow cytometry.

**Parvaquone and Buparvaquone degradation determined by Reversed-Phase High Performance Chromatography**
High Performance Liquid Chromatography (HPLC) was performed using an ÄKTA™ Avant system (GE Healthcare) coupled with a Kinetex C18, 5 µm particle size, 100A, 100 x 4.6 mm stainless steel column, manufactured by Phenomenex (Phenomenex Gemini). Mobile phases were 0.05 M Na-acetate buffer (pH 3.6)-Acetonitrile (35:65 v/v) and 0.05 M Na-acetate buffer (pH 3.6)-Acetonitrile (20:80, v/v) for BPQ and PQ, respectively. Elution was performed at 1 ml/min flow rate with an equilibration phase for 4 min followed by an elution step for 10 min for PQ and 8 min for BPQ. The protocols were adapted from Kinabo 1988 and Venkatesh 2007. Samples were injected using a 20 µl loop and detection was achieved by measuring simultaneously the UV absorption at 251 and 281 nm, with integration for determination of the area under the curve, which relates with the drug recovery. Integration was performed using the 251 nm chromatogram. Data collection and analysis was done using UNICORN™ 6.3 control software (GE Healthcare).

Calibration curves were constructed using the injectable drug due to unavailability of the pure compounds. The BPQ calibration curve was linear in the 20-1000 ng range and the PQ calibration curve was linear in the 30-1500 ng range. Parvaquone samples subjected to the different treatments were diluted 1/100 prior to injection while BPQ samples were diluted 1/200 in their respective mobile phase. Chosen dilutions ensured that samples that did not degrade fell well inside the linear range of the calibration curve, with only samples that went through complete degradation falling outside the linear range of detection.

Follow up experiment on the stability of buparvaquone and parvaquone exposed to air

Parvaquone and buparvaquone were diluted to 15 mg/mL and 5 mg/mL (respectively) in
N-methyl-2-pyrrolidone. Triplicate samples of 1.4 mL (completely filled) and 0.2 mL (partially filled) were prepared in brown 1.5 mL microcentrifuge tubes and left at room temperature for 50 days, protected from light. Samples included those with air exposure in the form of “headspace” in partially filled tubes, while the completely filled tubes had no obvious headspace. These samples were then evaluated for drug concentration using HPLC in comparison with a fresh, unexposed sample.

Data analysis

Mean PPE for each drug concentration and controls were calculated by averaging the PPE of three independent replicates. Percent of maximum PPE (% max PPE) was then calculated for each concentration using the mean PPE value in comparison to the highest PPE obtained in the assay for a given isolate. The 50% inhibitory concentrations (IC$_{50}$) were determined by fitting the curves with nonlinear regression and determining interpolated X values for Y=50, including 95% confidence intervals for a level of significance of $P<0.05$ (GraphPad Prism version 6.03, GraphPad Software, La Jolla, CA). The IC$_{50}$ values for each exposure replicate were compiled to calculate the mean, standard deviation, and % change in IC$_{50}$ (relative to the control of no exposure) for each exposure type and duration. Mean % max PPE values were also calculated to generate one mean IC$_{50}$ curve for each exposure type and duration to compare against a mean control curve to determine significance of each treatment compared to unexposed drug. Exposure replicates deemed significant outliers via Grubb’s test (http://graphpad.com/quickcalcs/Grubbs1.cfm) were eliminated from analysis.
Data from HPLC analysis was evaluated using Minitab 17 software (Minitab Inc., State College, Pennsylvania) by one-way ANOVA (F-test level of significance $P < 0.001$), using pairwise comparisons between each exposure duration for the content of each drug under each exposure condition. This was also performed to evaluate the relationships between all of the Air and Combo samples from each drug. Mean values were grouped according to Tukey HSC, simultaneous 95% confidence intervals. Average % of intact drug was calculated for each time point as a function of the Time 0 unexposed control. A nonlinear regression plot with one phase decay was then generated and Pearson’s correlation coefficient calculated to determine the relationship between these values and the corresponding mean IC$_{50}$ values; that is, depicting the correlation between drug content and efficacy.
RESULTS

In vitro susceptibility of T. equi to parvaquone and buparvaquone

Initial screening tests indicated that the IC$_{50}$ of unexposed BPQ and PQ were 0.0019 µg/mL and 0.0042 µg/mL, respectively. When this data was pooled with data from all experimental controls, the results remained very consistent with a mean IC$_{50}$ of 0.002 µg/mL (9.5% coefficient of variation) for unexposed BPQ and 0.0048 µg/mL mean IC$_{50}$ (9.0% coefficient of variation) for unexposed PQ. (Figure 1)

Effect of simulated environmental exposures on the in vitro susceptibility of T. equi to parvaquone and buparvaquone

Exposure to UV light and elevated temperature (37°C)

The mean IC$_{50}$ of triplicate BPQ samples with maximum exposures to ultraviolet light (UV$_{24h}$) and to elevated temperature (37°C$_{40exp}$) did not increase significantly compared to controls ($P>0.05$) based on the 95% confidence intervals, which all overlapped (Figure 2a). The same result was evident for PQ, and thus no further testing was pursued with respect to UV light or elevated temperature (Figure 2b).

Exposure to Air

Exposure of BPQ to air altered the IC$_{50}$ dramatically, with increases in the mean IC$_{50}$ for Air$_{14d}$, Air$_{30d}$, and Air$_{50d}$ of 538%, 1,531%, and 4,535% respectively ($P<0.05$) (Figure 3a). Air$_{7d}$ did not have a significant alteration in the IC$_{50}$ ($P>0.05$). The mean IC$_{50}$ of PQ had a significant
increase of 247% when exposed to air for 50 days (Figure 3b) but the increase of only 12.57% in Air\_30d was not significant. PQ Air\_14d and Air\_7d were not evaluated.

**Combination exposure**

Combination exposures were subjected to each of the following combinations of conditions: Combo\_7d – Five exposures 37°C/three hours UV light/seven days ambient air, Combo\_14d – 10 exposures 37°C/six hours UV light/14 days ambient air, Combo\_30d – 20 exposures 37°C/12 hours UV light/30 days ambient air, and Combo\_50d – 40 exposures 37°C/24 hours UV light/50 days ambient air (named according to length of air exposure). When exposed to multiple environmental conditions, the mean IC\textsubscript{50} of BPQ showed significant increases averaging 43% (BPQ Combo\_7d), 806% (BPQ Combo\_14d), 4233% (BPQ Combo\_30d), and 3526% (BPQ Combo\_50d) (Figure 4a). The IC\textsubscript{50} of PQ Combo\_50d increased an average of 93%, but due to the wide confidence interval for this exposure the result was not statistically significant. No significant change was found for PQ Combo\_30d or PQ Combo\_14d (Figure 4b). The shorter exposure of PQ Combo\_7d was not tested.

**Effect of simulated environmental exposures on the stability of parvaquone and buparvaquone**

**Exposure to UV light or elevated temperature (37°C)**

Buparvaquone exposed to 37°C for five or 20 exposures of eight hours each showed no change in drug concentration. The 37°C\textsubscript{5exp} samples showed a 20.65% decrease from the control ($P<0.05$), and the 37°C\textsubscript{10exp} treatment had less intact drug relative to the control.
(P<0.05) but not in comparison to 37°C_{C_{5exp}} or 37°C_{C_{20exp}} treatments (P>0.05). Ultraviolet light exposure caused a significant decrease in mean [BPQ] relative to the control in UV_{6h} and UV_{12h}, although the mean concentration in UV_{24h} did not differ. Overall, none of the exposure samples differed significantly from each other based on one-way ANOVA (P=0.017). (Figure 5a)

Parvaquone was not significantly degraded by exposure to 37°C for five, ten, 20, or 40 exposure periods of eight hours each (P=0.052). However, 24 h exposure to UV light resulted in a significant 11.90% decrease in the concentration of PQ relative to the unexposed sample. Exposure for 3, 6, and 12 h had no significant effect as compared to one another via one-way ANOVA (p=0.005). (Figure 5b)

*Exposure to ambient air*

The average concentration of BPQ underwent significant decreases of 34.09%, 88.53%, 95.25%, and 97.84% in Air_{7d}, Air_{14d}, Air_{30d}, and Air_{50d}, respectively (P<0.001) (Figure 5a). This degradation was time dependent, with all but BPQ Air_{30d} and Air_{50d} being significantly different from other treatment durations. Parvaquone stability was also affected by air exposure, but only Air_{30d} and Air_{50d} were associated with significant reductions in drug concentrations (23.46% and 68.82% respectively) (Figure 5b).

In a follow-up experiment, both drugs showed some degradation after 50 days of air exposure relative to an unexposed control, but the effect was only significant for BPQ (P<0.001). Partially filled tubes had a mean reduction in BPQ concentration of 45.17%, while the average reduction for the full tubes was 11% (Figure 6a). After 50 days, average PQ
concentration in partially filled containers was significantly lower than the control (15.37%) but was not significantly less than that of the filled tubes (7.23%) (Figure 6b).

**Combination exposure**

Exposure of BPQ to a combination of the aforementioned factors resulted in a significant decrease of the mean concentration of BPQ in all exposed samples ($P<0.001$). The average $\text{Combo}_{7d}$ concentration was 49.98% less than the control. Buparvaquone $\text{Combo}_{14d}$, $\text{Combo}_{30d}$, and $\text{Combo}_{50d}$ decreased by 86.23%, 96.43%, and 97.61% respectively, significantly less than the control but not from each other. (Figure 5a) Parvaquone $\text{Combo}_{14d}$, $\text{Combo}_{30d}$, and $\text{Combo}_{50d}$ were all significantly different from the unexposed control and from each other, with mean PQ concentration decreasing in a time dependent fashion by 11.61%, 77.51%, and 63.89% respectively from the Day 0 control (Figure 5b). The PQ $\text{Combo}_{7d}$ samples had a mean $[\text{PQ}]$ equaling 7.96% less than the control, but this difference was not statistically significant.

Comparison of all Air and Combo samples together revealed no difference between BPQ $\text{Air}_{50d}$, $\text{Combo}_{50d}$, $\text{Air}_{30d}$, $\text{Combo}_{30d}$, $\text{Air}_{14d}$, and $\text{Combo}_{14d}$ (data not shown). The only significant difference was found between BPQA$\text{Air}_{7d}$ and $\text{Combo}_{7d}$. No significant differences were found between paired PQ Air-Combo sample means at any time points (data not shown).

**Correlation of drug degradation with loss of efficacy**

As expected, the decrease in drug *in vitro* efficacy (represented by % increase in IC$_{50}$ against *T. equi*) was negatively correlated with % drug concentration for both BPQ ($r=-0.74, P=0.0093$) and PQ ($r=-0.88, P=0.0038$). A decrease in mean BPQ concentration up to 50%
resulted in a minimal loss of efficacy for the drug, but further degradation up to 90% caused a significant increase in IC$_{50}$. With greater than 90% degradation, IC$_{50}$ increased exponentially (Figure 6a). In PQ, a statistically significant change in IC$_{50}$ was not observed until the mean [PQ] had decreased by almost 69% (or [PQ] ~31% of the control) (Figure 6b). Degradation of more than 70% was not observed in this experiment.
DISCUSSION

The hydroxynaphthoquinone compounds investigated in this study, buparvaquone and parvaquone, are the current medications of choice for the treatment of East Coast Fever in cattle. Given that non-ideal storage conditions are probably the norm in East Africa, it is important to assess how exposure to extremes in temperature, UV, and ambient air might impact the efficacy of these drugs (Amin and Kokwaro, 2007). Whether by biological assay (T. equi susceptibility) or evaluation of drug degradation (HPLC), the only factor that had a statistically significant impact for both BPQ and PQ was exposure to ambient air. Overall, BPQ was less stable than PQ when exposed to ambient air for extended periods.

The decrease in efficacy for both drugs correlated well with drug degradation, albeit in a nonlinear fashion (Figure 7). With up to 50% degradation of BPQ, very minimal change in in vitro efficacy was observed. This is demonstrated in Air7d and Combo7d, which degraded significantly but showed no corresponding increase in IC50. Between 50 and 90% degradation, IC50 began to increase to a significant degree, and at more than 90% degradation IC50 increased exponentially. A similar relationship between drug concentration and IC50 was observed for PQ. As the only treatment with greater than 50% degradation, Air50d showed significant differences in both drug concentration and efficacy. However, the PQ concentration of Air30d, Combo14d, Combo30d, and Combo50d were significantly decreased with no effect on the mean IC50.

Further analysis of the HPLC data from Air and Combo samples determined that all of the degradation in BPQ Combo samples exposed to air for 14, 30, and 50 d could be accounted for by air exposure, with no additive or synergistic contribution from temperature and/or UV exposure. The only significant difference was found between Air7d and Combo7d. With the effect
of air not as overwhelmingly evident at this exposure duration, the effects from UV and temperature may have become additive. No differences were found for PQ samples, indicating that the degradation in Combo samples could be attributed to air exposure.

The stability of the drugs analyzed in this study is relevant and important to the practice of veterinary medicine in Africa. Compromised drug quality negatively affects the ability of veterinarians to control diseases such as ECF because effective management requires early and efficacious treatment with medications of dependable quality. One issue of great concern is the potential for development of drug resistance. At this time, resistance to naphthoquinones has not been widely reported in the veterinary literature, although resistance to BPQ has been observed and investigated in *T. annulata* (Mhadhbi et al., 2015; Mhadhbi et al., 2010; Sharifiyazdi et al., 2012). In human medicine, there is well documented resistance to the naphthoquinone drug atovaquone in apicomplexans such as *Plasmodium falciparum* (Cottrell et al., 2014), *Toxoplasma gondii* (McFadden et al., 2000), and *Pneumocystis carinii* (Kaneshiro, 2001).

Similar resistance has not yet been reported in *T. parva*, but prevention and treatment of ECF relies heavily on BPQ and PQ, with no viable and equivalent alternative options for therapy at the current time. As such, development of resistance to these drugs could be catastrophic. Even if a quality drug is obtained from a reputable source and administered properly, environmental exposure during transport or storage can result in significant deterioration of drug quality. Clinical use of these drugs exposed to conditions similar to that in this study would likely result in sub-therapeutic plasma levels in the treated animal, which is a well-known risk factor for development of drug resistance (Dzinjalamala et al., 2005; Nzila et al.,
Low levels of a drug apply selection pressure to the parasite population, selectively targeting highly susceptible organisms while allowing survival of more resistant parasites. This has been noted as a particular problem in drugs with a long half-life (Nzila et al., 2000; Watkins and Mosobo, 1993), which is particularly relevant in the case of BPQ. Therefore, the clinical impact of these findings in terms of both immediate effect (decreased efficacy for individual cases) and potential long term consequences (development of drug resistance) cannot be ignored. In both respects, compromised drug content and quality can result in overall increased treatment expense and animal suffering.
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FIGURES

Figure 1

a) Buparvaquone control IC₅₀: Overlaid control curves from all buparvaquone assays
b) Parvaquone control IC$_{50}$: Overlaid control curves from all parvaquone assays

Control IC$_{50}$ curves from all a) buparvaquone and b) parvaquone assays are overlaid to demonstrate the consistency of the control between assays
Figure 2: UV and 37°C exposure IC₅₀

a) Buparvaquone

Buparvaquone (a) and Parvaquone (b) samples are grouped by exposure condition (UV24h and 37°C x 40exp) and the IC₅₀ for controls (green circle) and each exposure replicate are plotted for comparison. Error bars represent the 95% confidence interval for each IC₅₀ as calculated by GraphPad Prism based on the goodness of fit of the growth inhibition curve.
Figure 3: Air exposure IC$_{50}$

a) Buparvaquone

All Air exposures

Day 2: % Inhibition

[BPO] µg/mL
b) Parvaquone

In the top panel for both a) buparvaquone and b) parvaquone, samples are grouped by air exposure duration and the IC_{50} for controls (green circle) and each exposure replicate plotted for comparison. Error bars represent the 95% confidence interval for each IC_{50} as calculated by GraphPad Prism based on the goodness of fit of the growth inhibition curve for each replicate.

Mean growth inhibition curves for each time point are plotted in the bottom panel to demonstrate the change in IC_{50} with increased duration of exposure. Light green represents the control, with peach (BPQ only), teal (BPQ only), brown, and blue representing 7, 14, 30, and 50 days of exposure, respectively. Curve shifts to the right are representative of an increase in IC_{50} value.
Figure 4: Combo exposures IC50s

a) Buparvaquone
b) Parvaquone

In the top panel for both a) buparvaquone and b) parvaquone, samples are grouped by exposure duration (based on the air component) and the IC\textsubscript{50} for controls (green circle) and each exposure replicate plotted for comparison. Error bars represent the 95% confidence interval for each IC\textsubscript{50} as calculated by GraphPad Prism based on the goodness of fit of the growth inhibition curve for each replicate.

Mean growth inhibition curves for each time point are plotted in the bottom panel to demonstrate the change in IC\textsubscript{50} with increased duration of exposure. Light green represents the control, with peach (BPQ only), teal (BPQ only), brown, and blue representing 7, 14, 30, and 50 days of exposure, respectively. Curve shifts to the right are representative of an increase in IC\textsubscript{50} value.
Figure 5: Concentrations of drug samples as determined by HPLC

a) Buparvaquone HPLC

b) Parvaquone HPLC

For both buparvaquone (a) and parvaquone (b), samples are grouped by condition on the x-axis. Time 0 control is represented by light blue, with orange, gray, yellow, and dark blue representing increasing exposures to 37°C (5, 10, 20, and 40 exposures) UV light (2, 6, 12, and 24 hours), Air (7, 14, 30, and 50 days) and Combinations of each of these exposure variables. The drug content in ng is plotted on the y-axis.
Figure 6: Follow up experiment for air exposure

a) Buparvaquone

For both buparvaquone (a) and parvaquone (b), samples types are present on the x-axis. Unexposed control (“Flask”) to the far left, and 50 days of exposure to minimal (middle) or increased air (right). The drug content in ng is plotted on the y-axis.
Figure 7: Correlation of % drug and decrease in drug efficacy

a) Buparvaquone

Calculated drug content as a % of the fresh control sample is plotted on the y-axis against the % of increase in IC$_{50}$ observed for a given sample in comparison to the control in order to demonstrate the correlation between drug degradation and decreased efficacy for a) buparvaquone and b) parvaquone.
CHAPTER THREE

INTRODUCTION

Theileria equi is a tick-borne apicomplexan parasite that causes the disease equine piroplasmosis, eradicated from the United States in 1988. Sporadic domestic outbreaks have occurred however, and there is a need for effective chemotherapeutics both in the United States and internationally. The most commonly used drug for treatment of T. equi is imidocarb dipropionate (IMD). Variation in susceptibility between parasite isolates has been demonstrated in vitro and incomplete clearance has been observed in vivo, which indicates that IMD resistance likely exists in T. equi at the parasite level [1-6]. It has also been shown in vitro that exposure to IMD results in emergence of resistance in a parasite population [1].

With most pathogens, the goal of treatment is to minimize the clinical impact of disease. Complete elimination of a pathogen is a considerable challenge, particularly when the host is unable to routinely clear infection independently. Imidocarb dipropionate is a dicationic diamidine of the carbanilide series of antiprotozoal compounds [7]. In one major U.S. outbreak localized in Texas [8], IMD successfully cleared over 160 naturally infected horses of T. equi (Dr. Angela Pelzel, USDA-APHIS, personal communication)[5,9]. Despite the overall success of treatment, there have been reported cases of treatment failure in both clinical and experimental infections without obvious clinical explanation. Failure of IMD treatment in T. equi-infected horses is characterized by incomplete parasite clearance and recrudescence of parasitemia following discontinuation of treatment. Importantly, failure of treatment with previously effective drug protocols in the human malarial agent Plasmodium falciparum is almost invariably associated with decreased in vitro susceptibility to the treatment drug [10].
The ongoing identification of evolving drug resistance in other Apicomplexan parasites such as *P. falciparum* necessitates active investigation of resistance mechanisms and continued development of novel drugs for effective treatment.

One potential mechanism of such resistance is via ATP binding cassette (ABC) transporters, which are important mediators of resistance in *Plasmodium falciparum* and other related organisms. Both ABC transporter gene copy number variations as well as single nucleotide polymorphisms (SNPs) have been associated with altered drug susceptibility. The most well-characterized mechanism of transporter-mediated resistance is through direct drug transport, effluxing the drug out of the organism or cell [11-17]. However, transporters can contribute to resistance in multiple ways.

Transporter proteins of the ABC family have been implicated in multidrug chemoresistance to a wide variety of therapeutic agents with significant variation in chemical structure. In mammals, tumor cells demonstrate resistance to numerous chemotherapy agents associated with the overexpression and mutation of ABC transporters, specifically multidrug resistance protein 1 (MDR1 or ABCB1), multidrug resistance-associated protein 1 (MRP1 or ABCC1), and breast cancer resistance protein (ABCG2) [18]. Antimicrobial resistance in bacteria, fungi, and parasites has also been linked to altered ABC transporter expression. Although *P. falciparum* resistance is generally multifactorial, the contributions of PfMRP1 and PfMDR1 transporters have been extensively characterized [19]. These two transporters are important mediators of resistance to multiple antimalarial drugs and represent genetic markers of drug resistant phenotypes. Both SNP mutations [20] and variation in gene copy number [13-17] have been implicated in alterations of *in vitro* drug susceptibility with additive effects and
subsequent failure of antimalarial therapy. Evaluation of the *T. equi* genome has identified 45 members of the ABC transporter family [21], more than any other hemoparasite genome sequenced to date, including *P. falciparum* with only 16 members. This includes sequences that share some amino acid sequence identity with PfMDR1, PfMDR2, and PfMRP1, with a total of 37 putative MRP-type (ABCC) genes and four MDR-type (ABCB) genes.

ABC transporter-mediated drug efflux and subsequent resistance occurs through direct binding of the drug as a substrate with subsequent transport out of the cell, reducing the amount of drug reaching the target. Parasitic ABC transporters described to date function almost exclusively in this manner, pumping substrates out of the cell or into intracellular organelles such as food vacuoles [18]. PfMRP1 is predominantly present in the plasma membrane, likely playing a role in drug efflux [22]. In contrast to human MDR1, PfMDR1 is primarily localized to the food vacuolar membrane, suggesting a function of pumping substrates into the food vacuole for digestion [23]. The implication of this localization for the effectiveness of various drugs differs however, as some drugs target metabolism that occurs within the food vacuole and would thus have increased efficacy if they were PfMDR1 substrates. This has been posited as the likely reason that PfMDR1 amplification and certain SNPs increase the sensitivity of *P. falciparum* to chloroquine and quinine, for which the food vacuole is the target site, but conversely decrease sensitivity to mefloquine and artemisinin [24-26].

Sensitivity to different drugs is affected differently by ABC transporter SNPs, probably because of altered binding affinity favoring one substrate drug at the expense of another [26,27]. The temporal and geographical dynamics of *P. falciparum* drug resistance strongly
support selection pressure as a driving factor for the emergence of specific SNPs and gene amplification. Followed over time, the prevalence of these genetic changes associated with resistance to a specific drug increases during treatment with that drug in a particular geographical region [20,28]. Specific SNPs associated with resistance to specific drugs also vary significantly between geographical areas, suggesting the independent emergence of mutations and supporting the multifactorial nature of resistance [16,20,28].

Pentamidine, a diamidine drug related to imidocarb, has been used for several decades to treat leishmaniasis and trypanosomiasis, although it has not been commonly used clinically for the treatment of malaria [29]. Toxicity and poor oral bioavailability have historically limited its use in favor of other antiprotozoals. In recent years, as *P. falciparum* has developed resistance to an increasingly wide array of antimalarial drugs, interest in diamidine compounds has been renewed [30]. There are multiple pentamidine derivatives currently in development and undergoing clinical testing as antimalarial alternatives, and these drugs have not demonstrated cross-resistance with currently used antiprotozoals [29,30]. However, ABC transporters have been implicated in resistance to pentamidine in both *Leishmania* and *Trypanosoma* species [31-33].

The mechanism of action of the diamidines is largely undefined. In murine *Trypanosoma brucei* infections, imidocarb appears to inhibit the uptake and/or metabolism of polyamines vital for cellular metabolism and DNA stabilization [34]. However, the uptake, cellular trafficking, and targeting of diamidine drugs in *P. falciparum* differs distinctly from other protozoan parasite species due to its intraerythrocytic nature, which likely impacts this process in *T. equi* as well. It has been demonstrated that pentamidine concentrates in infected
erythrocytes and is then taken up by malaria parasites via a “new permeability pathway” involving a plasma membrane choline carrier [29,35]. It binds to toxic products of hemoglobin digestion in the food vacuoles of erythrocyte stage *P. falciparum* parasites, inhibiting further metabolism and causing toxic levels of metabolite accumulation [29]. Many aspects of diamidine cellular trafficking have yet to be fully characterized but, given the ubiquity of ABC transporters, it is likely that one or more members of this family are involved. The purpose of this project is to investigate these ABC transporters as potential mediators of drug efflux and *T. equi* resistance to imidocarb dipropionate resulting in clinical treatment failure.

Characterizing mechanisms of drug resistance in *T. equi* allows for better prediction and protection of drug efficacy at the epidemiological and individual patient level. This in turn can aid in the prevention of progressive drug resistance and preserve the efficacy of available drugs, which is particularly important in large animal species as options for treatment are limited. Development of new treatment strategies, including novel drugs and combination therapy, will be better directed with this understanding. Ultimately, this research could support the implementation of successful treatment protocols as an alternative option for horses infected with *Theileria equi*, an outcome with significant impact both in the United States and worldwide.
METHODS

Evaluation of ABC transporter sequences in *Theileria equi*

Amino acid sequences encoding putative ABC transporters in the annotated *T. equi* genome were evaluated to verify the presence of the ABC transporter-specific sequence elements Walker A and Walker B domains and the ABC transporter signature motif in each gene (Figure 1). All full length transporters (~1400-1500 amino acids) were further classified into subtypes (ABCB and ABCC) based on conserved domains and sequence identity with other ABC transporters, determined by NCBI BLAST results ([http://blast.ncbi.nlm.nih.gov/Blast.cgi](http://blast.ncbi.nlm.nih.gov/Blast.cgi)). This allowed for subtype-specific selection of relevant ABC transporter inhibitors and substrates for use in subsequent assays.

*In vitro* parasite growth inhibition assay and determination of MK571 effect on IMD IC\(_{50}\)

The previously developed *in vitro* parasite growth inhibition assay for IMD susceptibility in *T. equi* [1] was adapted to incorporate MK-571, a broad spectrum inhibitor of ABCC subtype transporters. Briefly, two-fold serial dilutions of IMD ranging from 0.00094 \(\mu\)g/mL to 0.12 \(\mu\)g/mL were evaluated, with triplicate samples for each concentration, including infected erythrocytes and normal uninfected erythrocytes as controls with no IMD. The assay was performed in a 96-well plate over the course of 72 hours, with a target starting percent parasitized erythrocytes (PPE) for all wells of 0.3% and media changes at 24 and 48 hours. To evaluate the effect of MK-571 (50 \(\mu\)M), two separate assays were run in tandem using the same starting source sample FL USDA strain of *T. equi*. 
For assay initiation, 2x drug concentrations were prepared, with 100 μL of the appropriate concentration placed into each well with 100 μL of medium +/- 100 μM MK571 and 20 μL of RBCs to reach the final 1x IMD and MK571 concentrations for the assay and approximately 9% RBC concentration in each well. At 24 and 48 hours, 160 μL of medium containing the appropriate 1x IMD concentration was changed in each well, +/- 50 μM MK571, with plain medium used for controls. The assay was then evaluated at 72 hours by flow cytometry using hydroethidine staining to quantitate infected erythrocytes [1].

Mean percent parasitized erythrocytes (PPE) was calculated by averaging that of the three triplicate wells for each drug concentration and controls. Individual PPE values that deviated from the other replicates by >0.5% were considered outliers and eliminated from data evaluation. Percent of maximum PPE (% max PPE) was then calculated for each concentration using the mean PPE value in comparison to the highest PPE obtained in the assay for a given isolate. The 50% inhibitory concentrations (IC50) were determined by fitting the curves with nonlinear regression and determining interpolated X values for Y=50, including 95% confidence intervals for a level of significance of p<0.05. This was accomplished using GraphPad Prism version 6.03 for Windows (GraphPad Software, La Jolla, CA).

**Indirect transport assay using isolated *Theileria equi* merozoites**

Samples were harvested from ongoing *in vitro* cultivations of *T. equi*, with one well per sample (~125 μL of erythrocytes with ~8% PPE). Each sample was centrifuged at 500g for five min to pellet the erythrocytes, the culture media supernatant was removed, and they were washed with 1 mL of 1x PBS. Packed erythrocytes were then lysed with an equivalent volume
(125 µL) of 1x Red Blood Cell Lysis buffer containing ammonium chloride, potassium carbonate, and EDTA. After five minutes of incubation at room temperature the reaction was halted by adding 3x volume of 1x PBS (750 µL). The lysed solution was centrifuged at 400g for 10 min and the supernatant containing the merozoites transferred to a new tube. The merozoites were washed a final time in 1x PBS and pelleted by centrifugation at 4000g for 15 min.

For the indirect transport assay, triplicate samples (one well/sample) were utilized for each condition. Merozoites isolated by erythrocyte lysis were labeled via incubation with 1 µg/sample of anti-EMA antibody tagged using the Zenon AlexaFluor 647 nm Mouse IgG1 labeling kit (ThermoFisher Scientific). The merozoites were incubated with the antibody for 15 min at room temperature in the dark and then washed once with 1 mL of 1x PBS and centrifuged at 700g for 10 min. After removal of the supernatant, the merozoites were immediately incubated at 37°C for 60 min in one mL of either plain 1x PBS, 1 µg/mL IMD in PBS, or 50 µM MK571 in PBS. After this pre-incubation, a 5 mM solution of Carboxyfluorescein diacetate (CFDA) solubilized in DMSO (Life Technologies) was serially diluted in 1x PBS to 3 µM and added. The merozoites were then incubated for an additional 30 minutes in the dark at 37°C, followed by centrifugation at 700 g for 10 min and one wash with 1 mL of cold 1x PBS to remove exported substrate. Finally, they were suspended in 500 µL of cold 1X PBS containing 0.2% sodium azide for flow cytometric analysis.

Flow cytometry was performed similarly to evaluation of the in vitro growth inhibition assay, using a FACSCaliber flow cytometer equipped with CellQuest computer software (Becton Dickinson Immunocytometry Systems, San Jose, CA) on a Macintosh computer. Fluorescence profiles were recorded for later analysis with FCS Express software (De Novo software, Los
Angeles, CA). The EMA-labeled merozoites were picked up in the FL-4 channel while the CFDA registered in FL-1, allowing both parameters to be analyzed at the same time. Gating of the merozoite population was based off of EMA-labeled merozoites with no CFDA added (Figure 2), and 50,000 events per sample were collected. The data was analyzed in FCS Express to determine the mean CFDA fluorescence intensity of the labeled merozoite population. Mean and standard deviation were calculated from triplicate samples to determine the 95% confidence interval for each isolate in each condition.

**Generation of imidocarb dipropionate exposed parasites and replicates**

The methodology described below provided samples for which the presence of IMD was the only difference between exposed and unexposed parasites, eliminating as many confounding variables as possible in evaluation of differential transcription or function. The USDA FL *T. equi* strain (FLm) was used as the unexposed parent strain for RNA sequencing samples. Temporal replicates were derived by isolating RNA from FLm at three separate time points over the course of nine months (Figure 3). To generate exposed FL replicates (FLexp), one source well of the parent FLm strain was split 1:4 into three separate wells. After 24 hours, when the parasites were in the active growth phase with a PPE of approximately 4%, 1 mL of growth media was removed and replaced with 1 mL of media containing 0.008 μg/mL IMD (the IC₅₀ previously determined for this strain). Continuous exposure to increasing concentrations of IMD (0.008 μg/mL → 0.015 μg/mL → 0.03 μg/mL → 0.06 μg/mL → 0.1 μg/mL) was performed for each well, allowing parasite growth to recover to normal levels in between dosage increases. Recovery periods ranged from 2-3 weeks. In this manner, each of these triplicates
adapted to the presence of IMD independently to generate biological replicates for RNA sequencing (Figure 3). Three other *T. equi* isolates (FL2279, FL2280, and TX771) adapted into culture from chronically infected ponies were exposed to IMD in an identical manner to provide additional parent-exposed variant pairs for downstream evaluation.

**RNA sequencing processing and analysis**

Total RNA was isolated from the three temporal FLm replicates and three FLexp replicates exposed at a final concentration of 0.1 \( \mu \text{g/mL} \) (total of 142 days of continuous IMD exposure) using the Direct-zol RNA MiniPrep kit (ZymoResearch) with in-column DNase I digestion. Quality and concentration assessment was performed using a NanoDrop spectrophotometer (ThermoScientific) and samples were standardized to 50 ng/\( \mu \text{L} \) with nuclease free water. RNA was submitted to Fred Hutchinson Cancer Research Center at the University of Washington for next-generation RNA sequencing (RNA-Seq) on an Illumina HiSeq 2500 platform with 50 base paired end reads.

Image analysis and base calling was accomplished using Illumina's RTA v1.18.64 Software, with files generated in FASTQ format using Illumina's bcl2fastq v1.8.4 Software. Reads that did not pass Illumina's base call quality threshold were removed. Quality reads were aligned to the TequiWA genome (http://piroplasmadb.org/common/downloads/release-4.0/TequiWA) using TopHat v2.12 (http://ccb.jhu.edu/software/tophat/index.shtml). Bam files were sorted and indexed with samtools v0.1.19 (http://samtools.sourceforge.net/) and counts generated for each gene using htseq-count v0.6.1p1 in "intersectionstrict" overlapping mode.
(http://www-huber.embl.de/users/anders/HTSeq/doc/count.html). Genes without at least 1 count/million in at least 3 samples were removed from analysis.

Data was then normalized and compared using the exact test method in edgeR v3.6.8: (http://www.bioconductor.org/packages/2.14/bioc/html/edgeR.html). Genes were considered to be differentially transcribed between FLm and FLexp if the corrected p-value was less than 0.05 and the log fold change (log2 ratio) was greater than 0.585 or less than -0.585 (indicating greater than 1.5 fold change in transcription). Evaluation of this data was primarily focused on the *T. equi* ABC transporter family to determine differential transcription of these genes.

To identify SNPs present in the ABC transporter genes of IMD exposed versus unexposed *T. equi*, consensus transcript sequences were generated for each RNAseq replicate using the gffread utility in Cufflinks (https://github.com/cole-trapnell-lab/cufflinks). Multiple sequence alignment was performed using CLUSTALW within UGENE Unipro software package (http://ugene.net/download.html) and SNPs visually identified. Nucleotide sequences were then translated and aligned to compare amino acid sequences. Changes were visually observed after pretty-printing in BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html).

**Generation of BEWA_032300 transfected HEK293 cells**

Full length gene specific primers were designed using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) for all of the differentially transcribed ABC transporter genes based on the published genome sequence for *T. equi* (BEWA_032300 gene pictured in Figure 4). Both one-step and two-step RT-PCR and transformation of ABC transporter genes were attempted multiple times using RNA obtained
from several different cultured \textit{T. equi} isolates. Due to the length of the genes (~4.5 kb), results were very inconsistent and repeated failures occurred at different points of the process. Genomic DNA was therefore used as a source for PCR amplification, and efforts were focused on BEWA\_032300, the most differentially expressed putative ABC transporter and, as a significantly upregulated gene, the one most likely to be involved in drug efflux. Since no introns are present in \textit{T. equi} ABC transporter genes, this permitted accurate amplification of the appropriate full length gene without the added complicating factor of RT-PCR. Primers incorporating restriction enzyme sites allowed for direct ligation into the pcDNA3 vector (Figure 5) following restriction enzyme digest of the PCR product. One Shot\textsuperscript{®} TOP10 Chemically Competent \textit{E. coli} cells (ThermoFisher Scientific) were transformed with this vector and plated for subculture. Subculture colonies were propagated and DNA isolated with the Quick-gDNA miniprep kit from ZymoResearch. These products were then sequenced to verify amplification of the correct gene and the sequence of the cloned BEWA\_032300 gene matched with that of the published genome. The pcDNA-ligated plasmid containing the ABC transporter gene of interest and a selectable marker for Geneticin antimicrobial resistance was used to transfect HEK293 cells. Transfected cells were selected for through continuous cultivation in complete DMEM media with Geneticin (plus 10\% BSA and gentamicin). Transcription of the gene of interest was also verified in the transfected cell population via RT-PCR after three weeks of culture (Figure 6).
**Indirect transport assays with ABC transporter transfected HEK293 cells**

HEK293 cells transfected with the BEWA_032300 ABC transporter (HEK293-ABC) were initially evaluated for transport of CFDA and inhibition by MK-571. Calcein-AM, which is cleaved by intracellular esterases from a non-fluorescent precursor into brightly fluorescent calcein, was subsequently used as the fluorescent substrate as CFDA was not retained well in any cell type during the preliminary work. Additionally, the ABCC transporter inhibitor probenecid was also utilized as an alternative to MK571, in case previous results were due to an incompatibility with that particular inhibitor.

Prior to running a full triplicate assay with this combination, a preliminary investigation of incubation time and substrate concentrations was undertaken for assay optimization. Non-transfected HEK293 cells were plated in 12 well plates overnight and allowed to grow to 80-90% confluency. Growth media was vacuumed off and each well was gently washed with 1 mL of warm serum-free DMEM media, then replaced with serum-free media containing 0.1 or 0.5 μM calcein-AM. Plates were incubated with substrate only for 15, 20, and 30 minutes in the dark at 37°C. Media was again removed and replaced with warm serum- and substrate-free media, including probenecid (3 mM) if indicated. After 90 minutes of incubation for substrate efflux, the media was removed and cold complete media containing BSA was added to quench any remaining extracellular substrate. This was then vacuumed off and each well was washed once with cold 1x PBS. The cells were resuspended through vigorous pipetting in 200 μL of cold 1X PBS for flow cytometric analysis, with evaluation of fluorescence in the FL-1 channel.

Cells transfected with the Equine Infectious Anemia envelope gene (env) served as the negative control in further evaluation of calcein-AM as a substrate and probenecid as an
inhibitor. After optimization, IMD transport by this transporter was assessed using an indirect competitive inhibition assay. Triplicate wells were assessed for each cell type in each condition: No substrate, Calcein efflux only (0.5 µM), Calcein + Probenecid efflux (2 mM), and Calcein + IMD efflux (100 µg/mL), all diluted in serum-free DMEM media. The assay was performed as described above, and data was analyzed as for the merozoite transport assays.
RESULTS

Imidocarb dipropionate susceptibility of *T. equi* with and without MK571

The application of 50 μM MK571 increased the IC$_{50}$ the FLm strain from 0.01 μg/mL to 0.05 μg/mL, a significant five-fold change (P<0.05) (Figure 7a). The almost eight-fold change from 0.007 μg/mL to 0.054 μg/mL in FL2279 was also statistically significant (P<0.05) (Figure 7b). Baseline IC$_{50}$ of FL2279exp was much higher (0.140 μg/mL), however it did not change significantly with the addition of MK571 (0.188 μg/mL, P>0.05).

Indirect imidocarb dipropionate transport assay in isolated merozoites

Although the fluorescent substrate was readily taken up and merozoites labeled well with anti-EMA antibody, no difference in mean fluorescence intensity was observed between FL2279 and FL2279exp or between FL2280 and FL2280exp (Figure 8). This was true as well when examining the effects of adding MK571 or IMD. No shift in peak fluorescence could be appreciated with any experimental treatment (p>0.05).

Differential transcription and single nucleotide polymorphisms in putative *T. equi* ABC transporters as identified by RNA sequencing

Eight ABC transporters were identified as being differentially expressed, with three upregulated and five downregulated in FLexp parasites (Table 1). Of these eight, two (one upregulated – BEWA_037280, and one downregulated – BEWA_054480) were immediately eliminated from further evaluation due to anomalous values in one of the three triplicates that led to false significance. Three non-synonymous SNPs were also identified in the most highly
upregulated ABC transporter (BEWA_032300), present in all three of the FLexp replicates relative to the FLm samples and reference transcriptome. All of these SNPs result in missense amino acid substitutions (Table 2), with the potential for significant effect on protein structure and substrate binding. Based on these changes, the significantly upregulated BEWA_032300 became the focus of further isolation and evaluation in subsequent assays. Two other non-synonymous SNPs of interest creating missense amino acid substitutions in all FLexp replicates were found in other ABC transporters, BEWA_022390 and BEWA_022760 (Table 2).

Effect of inhibition by MK571 or probenecid and application of imidocarb dipropionate on fluorescent substrate accumulation in BEWA_032300 transfected and control HEK293 cells

HEK293-ABC cells accumulated a slightly higher amount of CFDA than the negative control both with substrate alone (p=0.041) and with IMD (p=.028) (Figure 9). The inclusion of MK571 resulted in a huge increase in substrate accumulation in both cell types, however the transfected cells accumulated significantly less overall (p=0.022). Modest increases in CFDA accumulation were also observed with IMD in both cell types, but the increases were of a consistent degree between the control and the transfected cells.

Calcein was well retained in all cell types (Figures 10 & 11), and in Figure 10 the minimal effects of varied substrate concentration and incubation duration are pictured, as well as the lack of effect observed with probenecid only. There was no significant change in substrate accumulation with any treatment in the control HEK293-env cells, and accumulation in HEK293-ABC cells was significantly higher at all time points (p<0.05) (Figure 11). With the addition of
probenecid, accumulation in HEK293-ABC cells increased to a significant degree, unlike HEK293-env cells. Addition of high concentration IMD had no effect.
DISCUSSION

Imidocarb dipropionate is a diamidine compound that is currently one of the only clinically feasible drugs demonstrating efficacy for clearance of T. equi infection [5]. Failure of IMD treatment has been reported previously, and variation in IMD susceptibility demonstrated between T. equi isolates in vitro [36]. The existence of imidocarb-specific [6] and multidrug resistance [13-15,37] in related parasites raises significant concern over the existence of drug resistance in T. equi, particularly emergence in response to antimicrobial exposure. In the human parasite P. falciparum, ABC transporters contribute significantly to drug resistance against current antimalarials [15,17,19,25,26,28,38].

In the current study, RNA-Seq was utilized to generate transcriptome profiles for comparison between different T. equi isolates, specifically those subjected to in vitro imidocarb selection pressure. This facilitated identification of genetic variability and differential transcription within the putative 45 member ABC transporter family associated with resistance phenotype. Six ABC transporters of potential import with significant differences in level of gene expression were identified through this methodology, including the highly upregulated BEWA_032300 with a logFC value >3. This equates to BEWA_032300 being transcribed at a level eight times higher in the FLexp parasite populations than in the parent FLm strain. These results are in the process of being validated using quantitative real time RT-PCR in multiple parent-exposed variant pairs to provide additional evidence for the importance of this genetic change in resistance to IMD.

This same gene also demonstrated several non-synonymous SNP mutations, including one that resulted in an amino acid substitution at residue 491 (D → G), a second causing an (A
\( \rightarrow S \) substitution at amino acid 988, and a third substituting (I \( \rightarrow \) V) at residue 1435 in all FLexp replicates. Another putative ABC transporter gene without differential expression (BEWA_022390) possessed a SNP causing a D \( \rightarrow \) G amino acid substitution in a similar location to BEWA_032300 (residue 498) in all three of the FLexp triplicates. One other SNP of potential interest was identified in a third putative ABC transporter gene, BEWA_022760, causing a V \( \rightarrow \) I amino acid substitution at amino acid residue 541.

SNPs can cause alterations in substrate binding affinity, transport function, ATPase activity, ATP binding activity, and protein 3D structure among other things, all of which could affect drug transport [19,27,28,38-40]. Interestingly, parasite sensitivity to different drugs is affected in different ways by ABC transporter SNPs in \( P. falciparum \), likely due to altered binding affinity favoring one substrate drug at the expense of another [26,27]. The two D \( \rightarrow \) G substitutions described here in particular have the potential to significantly alter the 3D protein structure and binding properties of the affected ABC transporters. Aspartate (D) is a negatively charged, polar amino acid which generally functions to either interact with an aqueous environment or create salt-bridges with other, positively charged amino acids important for protein stability [41]. In contrast, glycine (G) is hydrophobic and the smallest amino acid. It is often present within loops and is generally highly conserved due to its importance for preserving 3D protein structure. Substitution with glycine tends to increase protein flexibility and as a hydrophobic amino acid it does not typically reside in an aqueous environment [41].

Substitution of serine (S) for alanine (A) also has the potential to affect substrate recognition or specificity, as alanine is nonpolar and very non-reactive, whereas serine is polar, with a highly reactive hydroxyl group that readily forms hydrogen bonds with polar substrates.
The V → I substitutions are likely less influential, as both amino acids are neutral, nonpolar, and hydrophobic [41]. They can be involved in substrate recognition of hydrophobic ligands, which could be a characteristic of interest.

This study also investigated *T. equi* ABC transporters from a functional perspective. The results derived from this series of experiments were unexpected. Typical ABC transporter-mediated drug resistance mechanisms that have been described in the literature occur through direct binding of the drug in question by a transporter at the plasma membrane with subsequent efflux of the drug out of the cell. An inhibitor of this process (such as MK571) would be expected to further sensitize the cell (or parasite) to the drug due to increased drug accumulation. Inhibitors such as this have been shown to actually reverse ABC transporter-mediated resistance [18,40,42]. Instead, we saw a clear opposite effect with the addition of MK-571 in the *in vitro* parasite growth inhibition assay for IMD susceptibility. The IC$_{50}$ of the two non-IMD exposed isolates examined was actually significantly increased in the presence of MK571, indicating increased IMD resistance. The fact that no significant change was observed in the IMD adapted FL2279exp variant raises the question of whether the mechanism of drug resistance in this variant is through a mechanism related to ABC transporter inhibition (i.e. ABC transporter-mediated via different route than drug efflux).

Results of the merozoite indirect IMD transport assay also did not support IMD as a substrate for ABC transporters effluxing the drug from the parasite, as occurs in *P. falciparum* and many other drug resistant organisms and cell types. There was no significant difference in accumulation of the fluorescent ABCC transporter substrate CFDA between control parasites exposed to CFDA only and those exposed to IMD or the ABCC inhibitor MK571, despite the fact
that ABCC subtype transporters represent the vast majority of ABC transporters identified in the *T. equi* genome. This means that substrate egress from merozoites was neither inhibited directly by MK571 nor inhibited by IMD competing with the substrate for efflux from the cell. There was also no significant difference observed between IMD adapted parasites from FL2279exp and FL2280exp and the more susceptible parent isolates FL2279 and FL2280. These organisms clearly took up substrate and converted the CFDA ester to its active fluorescent form carboxyfluorescein as fluorescence was increased relative to no substrate. Parasitic transporters either did not bind the fluorescent substrate, transporters from all isolates transported it with equal efficiency, or all trafficking of the substrate occurred intracellularly. These findings could indicate that the substrate and/or inhibitor are not ideally compatible with transporters in *T. equi* and alternatives should be pursued. Although many different variables have already been evaluated, additional optimization of this assay for incubation times or drug concentrations may be possible.

The merozoite assays allowed us to examine the entire cast of ABC transporters present in *T. equi* as a functioning unit, but in order to pinpoint the specific role of an ABC transporter such as BEWA_032300, the individual transporter had to be isolated and transfected into a cell type with minimal endogenous transporter function of its own (such as HEK293 cells). After successful transfection (verified through RT-PCR of the transfected cell population – Figure 5) the indirect IMD transport assay was performed using transfected cells to assess this isolated transporter. Incubation with only CFDA resulted in very low accumulation in both control cells and HEK293-ABC cells, which was increased dramatically through the application of MK571. However, the effect was significantly greater in control cells than HEK293-ABC cells, which is
not consistent with the mechanism of the BEWA_032300 transporter becoming blocked from effluxing substrate. With IMD added, CFDA accumulation was very mildly increased, again in both cell types but this time to a similar degree. This does not indicate that IMD is competing with the substrate for efflux from the cell via the BEWA_032300 ABC transporter, since no significant difference was observed between control cells and those transfected with the gene of interest.

Calcein-AM and probenecid were tested as potential alternative reagents for this assay. This substrate was retained much better in the cell, with high levels even after the time allowed for efflux, eliminating the concern of passive substrate leakage contributing to assay variability. There was no significant change in substrate accumulation with any treatment in the control HEK293-env cells, which is the expected result. However, accumulation in HEK293-ABC cells was significantly higher at all time points. The reason behind this is unknown, but after addition of probenecid, accumulation in HEK293-ABC cells increased to a significant degree, unlike HEK293-env cells. This supports efflux of the substrate by the transporter becoming inhibited, as was the predicted result for this assay. However, the addition of high concentration IMD had no effect.

All of the presented evidence supports that the transfected ABC transporter is not efficiently binding IMD as a substrate and exporting it out of the cell as has typically been described for ABC transporter-mediated drug resistance. The transfected transporter sequence was isolated from the parent FLm strain and not the IMD exposed variant, so it is possible that the mutated and upregulated transporter present in exposed parasites binds more efficiently to IMD for transport. Given the significant upregulation of this transporter, it is likely involved in
resistance, but via a different mechanism than direct drug efflux. Potential possibilities for upregulated transporters include export of toxic products resulting from IMD action or a normal physiologic function of the transporter that counteracts the action of IMD. Downregulated transporters may be involved in uptake of IMD or intracellular transport of IMD to its unknown site of action. They also can be related in a compensatory manner, downregulating cellular processes that would normally support the action of IMD. Further evaluation of other identified differentially regulated ABC transporters for their importance in resistance phenotype will be undertaken through the described system in order to determine if any are involved in IMD trafficking. In the case of transporters with SNP mutations (including BEWA_032300) this will include comparison of transporters isolated from both IMD exposed and unexposed isolates.

Examination of the other differentially transcribed and annotated genes has identified several other genes of interest, in addition to the described ABC transporters. First of all, the uptake of pentamidine through the plasma membrane in *P. falciparum* occurs via a choline carrier. This may also be true in *T. equi*, as a gene encoding a putative choline kinase (BEWA_045430) is one of the most significantly downregulated genes. Subsequent decreased uptake of IMD would of course increase resistance.

Several of these genes also tie together in a potential resistance scheme that may describe a role for multiple ABC transporters as well. Although the mechanism(s) of action for IMD are currently unknown, the related drug pentamidine has been shown to inhibit digestion of hemoglobin in the food vacuole of *P. falciparum* leading to the accumulation of toxic metabolites. *T. equi* ultrastructure includes food vacuoles, inside of which a hemoglobin-like
substance can be visualized on electron microscopy [43-45]. Therefore, one mechanism for IMD similar to pentamidin can be theorized.

In this model, blockage of heme digestion by IMD results in accumulation of oxidized iron products. Unmitigated, this eventually leads to parasite death. However, if heme digestion is downregulated and toxic products are transported from the food vacuole to the cytosol for detoxification by reduced glutathione, the parasite can survive drug exposure. *T. equi* possesses five annotated cysteine protease family member proteins (BEWA_020560, 040480, 000590, 040110, and 018230), all of which are significantly downregulated in IMD exposed parasites. In *P. falciparum*, several of these genes are dedicated hemoglobinases, responsible for the digestion of host hemoglobin. In the proposed mechanism for IMD described above, it would follow that decreasing hemoglobin digestion would increase IMD resistance as a decreased amount of toxic products would be formed. A cytochrome B5 heme binding domain containing protein is significantly upregulated (BEWA_034670), which is generally involved in reducing oxidized methemoglobin. Toxic heme products cause the production of reactive oxygen species, which in turn oxidize hemoglobin to methemoglobin. Upregulated cytochrome B5 could help compensate for this effect and decrease the overall oxidative burden on the parasite.

This also suggests a role for the BEWA_032300 ABC transporter. It contains a conserved FepC domain, generally present as a part of an ABC-type Fe$^{3+}$ siderophore transport system. This protein is potentially present in the wall of the food vacuole, transporting toxic Fe$^{3+}$ heme out into the cytosol for degradation, explaining why it would by upregulated in exposed parasites. Additionally, glutathione is a known substrate for ABCC1 in multiple organisms [39]. It is quite possible that one of the downregulated ABC transporters is responsible for glutathione
export, as it would be advantageous to increase the intracellular glutathione concentration to assist in heme detoxification. As is the case with pfMDR1 and its role in the uptake of chloroquine and other drugs into the food vacuole \([16,17,24-27,38]\), an ABC transporter may also be responsible for the transport of IMD into the food vacuole. Consequently, downregulation (or inhibition) of such a transporter would increase resistance to the drug, if in fact IMD targets hemoglobin digestion in this location.

*Theileria equi* has many unique genes that could not be annotated through comparison to other organisms and cell types \([21]\). This complicated global analysis of our RNA seq data, since many of the genes that were differentially expressed were minimally annotated. Despite follow up analysis to attempt further classification. No orthologues to identified genes with a known role in drug resistance could be identified. This data could still be very useful for further investigation as additional genes are characterized and potentially as genetic markers of resistance.
REFERENCES


### Table 1: Differentially transcribed putative ABC transporters in *T. equi*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Log fold change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upregulated</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BEWA_032300</td>
<td>3.013</td>
<td>1.93E-120</td>
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<tr>
<td>BEWA_010780</td>
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</tr>
<tr>
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<tr>
<td><strong>Downregulated</strong></td>
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<td></td>
</tr>
<tr>
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<tr>
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Table 2: Amino acid substitutions in ABC transporters resulting from identified SNPs

<table>
<thead>
<tr>
<th>Gene</th>
<th>AA</th>
<th>From</th>
<th>To</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEWA_032300</td>
<td>491</td>
<td>D (aspartate)</td>
<td>G (glycine)</td>
</tr>
<tr>
<td></td>
<td>988</td>
<td>A (alanine)</td>
<td>S (serine)</td>
</tr>
<tr>
<td></td>
<td>1435</td>
<td>I (isoleucine)</td>
<td>V (valine)</td>
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<tr>
<td>BEWA_022390</td>
<td>498</td>
<td>D (aspartate)</td>
<td>G (glycine)</td>
</tr>
<tr>
<td>BEWA_022760</td>
<td>541</td>
<td>V (valine)</td>
<td>I (isoleucine)</td>
</tr>
</tbody>
</table>
FIGURES

Figure 1: Representative ABC transporter BEWA_032300

Conserved ABC transporter domains are identified, including ATP binding sites, ABC transporter signature motifs, Walker A/P loops, and Walker B sequences.
Figure 2: Flow cytometric gating of isolated merozoites

a) **Control 2279 (ema-label only)** Inclusion gating for “lysed RBC” population containing merozoites, based on forward (x-axis) and side (y-axis) scatter characteristics.

b) **Control 2279 (ema-label only)** Selection of ema-labeled merozoites from gated “lysed RBC” population based on FL-4 fluorescence (x-axis) plotted against side scatter.

c) **Control 2279 (ema-label only)** Quadrant gating of “lysed RBC” population, x-axis=CFDA (FL-1 fluorescence), y-axis=ema. Dual labelled cells gated in upper right quadrant.

d) **2279 (ema + CFDA)** “lysed RBC” population, x-axis=CFDA, y-axis=ema. Dual labelled cells gated in upper right quadrant.

e) **Control 2279 (ema-label only)** plot represents isolated “merozoite” population from 2a, with x-axis=CFDA to gate CFDA-containing merozoites in the lower right quadrant.

f) **2279 (ema + CFDA)** “merozoite” population, CFDA-containing merozoites in the lower right quadrant.
Samples of the FL maintenance strain were isolated as temporal replicates, and at a single time point three samples were split from the parent strain into separate wells to undergo independent exposure to imidocarb dipropionate over time, resulting in the biological triplicates for the purposes of RNA sequencing.
Figure 4: Primers and real time PCR probes for BEWA_032300

a)

Figure 4: Primers and real time PCR probes for BEWA_032300

b)
c) Mapped locations of full length primers for RT and detection primers and probes for real time PCR for putative ABC transporter gene BEWA_032300
   a) Locations in gene
   b) Locations and sequence at the nucleotide level (5’ end)
   c) Locations and sequence at the nucleotide level (3’ end)
Figure 5: Map of the pcDNA3\textsuperscript{TM} vector (Invitrogen)

Figure 6: RT-PCR verification of BEWA\_032300 gene transcription in transfected HEK293 cells using gene specific detection primers

Lane 1: Positive control (DNA isolated from cultured \textit{T. equi} merozoites)
Lane 2: Negative control (water)
Lane 3: pcDNA\_032300 transfected 293 cells (with reverse transcriptase)
Lane 4: Negative control – pcDNA\_032300 transfected 293 cells (no reverse transcriptase)
Lane 5: Negative control – pcDNA\_EIA\_Env transfected 293 cells (with reverse transcriptase)
Lane 6: Negative control – pcDNA\_EIA\_Env transfected 293 cells (no reverse transcriptase)
Figure 7: The effect of ABC transporter inhibitor MK571 on imidocarb dipropionate susceptibility in FLm, FL2279, and the imidocarb exposed variant FL2297exp

a) FLm (red triangles) & FLm + MK571 (purple diamonds), increase in IC$_{50}$ with the addition of MK571 indicated by the black arrow

b) FL2279 (red triangles) & FL2279+MK571 (purple diamonds), increase in IC$_{50}$ with the addition of MK571 indicated by the black arrow

FL2279exp (green circles) & FL2279 exp+MK571 (blue squares), increase in IC$_{50}$ with the addition of MK571 indicated by the black arrowhead
Figure 8: Mean fluorescence intensity of isolated merozoites in merozoite transport assay

![Merozoite transport assay](image)

Different isolates represented in separate groups on the x-axis, including two parent-imidocarb exposed pairs (2279-2279 exp and 2280-2280 exp)

- Blue = Fluorescent substrate only (2 μM CFDA)
- Red = 2 μM CFDA + 50 μM MK571
- Green = 2 μM CFDA + 1 μg/mL imidocarb dipropionate
Figure 9: CFDA efflux by HEK293 and HEK293-ABC cells

Blue = non-transfected HEK293 cells
Red = HEK293-ABC cells (transfected with BEWA_032300 ABC transporter)

“No CFDA”= negative control
“CFDA efflux”= incubated with CFDA only (5 μM)
“CFDA efflux + MK571 pre”= CFDA + ABCC transporter inhibitor MK571 (50 μM)
“CFDA efflux + IMD pre”= CFDA + imidocarb dipropionate (60 μg/mL)
Figure 10: Calcein efflux from non-transfected HEK293 cells – concentration and duration test

a) Inclusion gating for HEK293 cell population, forward scatter (x-axis) vs. side scatter (y-axis)
b) 2D plot for HEK293 control, no substrate. X-axis represents calcein (FL-1) fluorescence.
c) 2D plot for HEK293 + 3 mM probenecid only

d) 0.1 µM calcein x 15 min incubation
e) 0.5 µM calcein x 15 min incubation
f) 0.1 µM calcein x 20 min incubation
g) 0.1 µM calcein x 20 min incubation + Probenecid 3 mM during efflux
h) 0.1 µM calcein x 30 min incubation
Figure 11: Calcein efflux from HEK293-env and HEK293-ABC cells

Blue = HEK293-env cells (transfected with EIA-env protein)
Red = HEK293-ABC cells (transfected with BEWA_032300 ABC transporter)

“No substrate” = negative control with no calcein
“Calcein efflux” = incubated with calcein-AM only (0.5 μM)
“Calcein efflux + probenecid” = Calcein-AM with ABCC transporter inhibitor probenecid (2 mM)
“Calcein efflux + IMD” = Calcein-AM with imidocarb dipropionate (100 μg/mL)

* Significant difference between BEWA_032300 transfectants and env control (p<0.05)
** Significant difference in BEWA_032300 with probenecid added versus calcein only (p<0.05)
GENERAL CONCLUSION

The existence and progressive development of drug resistance in apicomplexan organisms represents a potential major complicating factor for treatment at both the individual patient and population levels in human and veterinary medicine. This can only be surmounted by careful stewardship of our existing antimicrobial repertoire and continually striving to understand underlying mechanisms of resistance. To that end, the work described here has approached the issue from multiple angles in several diseases of great global import.

Parasite elimination from *T. equi* infected horses has recently been demonstrated as a reasonable goal in the aftermath of the widespread U.S. outbreak in 2009. However, the demonstrated variability in imidocarb susceptibility and lack of alternative drug options makes this approach unfortunately unreliable at the current time. As has been characterized in numerous other organisms, including *P. falciparum*, ABC transporters may play a pivotal role and are worthy of thorough investigation, particularly since so many putative ABC transporter family members have been identified in the *T. equi* genome. This investigation is a large undertaking that is currently ongoing, with additional experiments to come in the near future that build on the solid foundation presented here. The described research has made key contributions to this process through genetic evaluation and the development of tools for functional assessment of transporters, along with extensive work in drug susceptibility testing, parasite cultivation, and generation of drug exposed variants utilized in a whole host of assays.

Development of alternative drug options can be guided by this understanding, as methods to avoid or modulate ABC transporter-mediated resistance can be pursued. Further investigation of drug approaches with novel mechanisms of action, such as the experimental
bumped-kinase inhibitors, may circumvent the resistance problems encountered with the use of imidocarb dipropionate. Likewise, incorporation of clinically relevant ABC-transporter inhibitors in a combination therapy approach may allow continued use of currently available drug options with improved efficacy. The identification or elimination of *T. equi* ABC transporters as mediators of drug resistance provides vital information in the development of sanctioned chemotherapeutic options for control of *T. equi* in the United States, with benefits for the horse industry both domestically and internationally.

Drug resistance concerns are certainly not unique to *T. equi* – as presented here, this is a particular consideration for *T. parva* as well, as ECF is another disease for which there are limited treatment options. The economic and practical concerns of production medicine represent a considerable challenge even in ideal circumstances. Practitioners in Sub-Saharan Africa face the additional difficulties of disease management in rural and impoverished areas oftentimes under adverse conditions.

The impact of drug resistance research and investigation of alternative therapies is not limited to the US – theilerial diseases are a worldwide problem and treatment is notoriously difficult. In veterinary medicine, particularly large animal practice, very limited drug options exist, including those for antimicrobial therapy. Practical considerations present significant limitations to availability and use, compared to humans and even small animals. Preservation of drug efficacy is therefore of paramount importance, especially for diseases of such economic and international importance for which no effective and practical treatment alternatives exist. Maintaining drug quality, understanding potential mechanisms of resistance, and investigating new therapeutic options are all key factors in pursuit of this objective.