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Efficacy of imidocarb dipropionate in eliminating *Theileria equi* from experimentally infected horses

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ABSTRACT

Theileria equi, one of the causative agents of equine piroplasmosis, is endemic in many regions of the world but is considered a 'foreign' animal disease in the USA. In an effort to prevent the importation of *T. equi*, stringent serological screening of horses is practiced prior to entry to the USA. Current regulatory options available where horses are found to be infected include permanent quarantine with or without chemotherapy, repatriation, or euthanasia. Chemotherapeutics that eliminate infection and subsequently transmission risk are critical in the management of infected horses. In this study, the efficacy of the drug imidocarb dipropionate against experimental *T. equi* infection was assessed.

Of nine horses experimentally inoculated with *T. equi* isolated from an animal previously imported from Peru, six were treated with imidocarb dipropionate after the resolution of the acute phase of the disease. Elimination of the parasite was demonstrated in 5/6 by nested PCR, blood transfusions to naïve horses, and reversion to seronegative status. The findings support the use of this drug as a potential treatment option in controlling outbreaks of *T. equi*, and also suggest that 'combination testing' using both serological and PCR detection methods are necessary to demonstrate clearance of infection.

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Introduction

Theileria equi, formerly of the genus *Babesia*, along with *Babesia caballi* (Mehlhorn and Schein, 1998), are the two causative agents of equine piroplasmosis. A member of the phylum Apicomplexa, *T. equi* is a protozoa transmitted naturally to horses by biting ticks or iatrogenically. Given that the distribution of *T. equi* infection is dependent on competent tick vectors of the genera *Rhiphicephalus*, *Hyalomma*, and *Dermacentor*, infection is endemic in Asia, Southern Europe, Latin America, and Africa (Ristic, 1988). It has been estimated that up to 90% of the world's equine population resides in areas where *T. equi* is endemic (de Waal, 1992). Following transmission from ticks, the acute phase of the disease in an immunologically naïve horse is characterized by fever, icterus, anemia, and hemoglobinuria (Schein, 1988). Following the resolution of the initial clinical signs, horses remain carriers for life and are reservoirs for further transmission (Holbrook, 1969; Ueti et al., 2008).

As equine piroplasmosis is considered exotic to the USA, a stringent policy involving the serological screening of all horses for both etiological agents prior to their entry of the county is practiced. Despite such procedures, infected carrier animals have gained, and are likely to continue to gain, entry (Short et al., 2012). When infected horses are detected in non-endemic regions or countries elimination of infection is essential. The US Veterinary Services Memorandum 555.20 states that when infection with *T. equi* or *B. caballi* is detected in the USA, regulatory actions available to owners of infected domestic horses include (1) permanent quarantine that may include chemotherapy, (2) exportation, or (3) euthanasia.

Chemotherapy has been approved as an option in only a small number of selected cases, in part due to previously published studies, which demonstrated differences in efficacy of imidocarb dipropionate. Frerichs et al. (1973), successfully eliminated infection from 13/14 horses using a dose of 4 mg/kg IM on four occasions at 72 h intervals. Clearance of infection in that study was demonstrated as failure of recipient ponies to seroconvert by complement fixation test (CFT) following the inoculation of blood from the treated horses. However Kuttler et al. (1987) observed that the same dosage of imidocarb dipropionate did not eliminate *T. equi* infection from six geldings as demonstrated by the observation of parasites in splenectomized recipient horses following their inoculation with blood from the treated animals.

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More recently Butler et al. (2008) determined that five doses of 4.7 mg/kg imidocarb dipropionate administered IM at 72 h intervals failed to eliminate *B. caballi* and *T. equi* from naturally infected carrier horses as indicated by the detection of parasites using PCR combined with reverse-line blotting. But Schwint et al. (2009) were successful in eliminating *B. caballi* infection from experimentally infected horses using imidocarb dipropionate treatment; the effectiveness of the treatment was assessed by the inability to transmit infection to recipient horses by either sub-inoculation of blood or tick transmission as determined by nested PCR (nPCR) and competitive ELISA (cELISA).

The objective of the current study was to determine if imidocarb dipropionate is capable of eliminating *T. equi* from experimentally infected horses. For the purposes of this experiment, success in eliminating the parasite was defined as: (1) the inability to detect *T. equi*-specific DNA by nPCR on at least three consecutive blood collections; (2) failure to establish infection in a naïve horse following transfusion of 100 mL of blood from treated animals; and (3) reversion of the experimentally infected horses to seronegativity following treatment. We expanded on previous, similar treatment studies by employing the use of sensitive molecular detection assays and in correlating the results of these assays with the expensive and arduous task of sub-inoculation. The possibility that treatment with imidocarb dipropionate could result in false negative readings in animals tested using the 'regulatory' cELISA was also examined.

Materials and methods

Animal selection and inoculum preparation

Twenty-one naïve mixed-breed horses free of *T. equi* infection as determined by cELISA, CFT, and nPCR were housed as two separate groups, namely, inoculated and negative control animals. All experimental procedures were approved by the NVSL/CVB Institutional Animal Care and Use Committee under Animal Care and Use Protocols 1785 and APHIS-2190.

The source of inoculum was a naturally-infected Thoroughbred horse imported from Peru. One hundred milliliters of blood were given IV to a splenectomized horse in order to generate high levels of *T. equi* in its peripheral blood. In brief, 500 mL of blood were collected in acid citrate dextrose and centrifuged at 900 *g* for 10 min. The buffy coat and plasma were removed, and the packed cells were washed once with Alsever's solution (20.5 g glucose, 8.0 g sodium citrate, 4.2 g sodium chloride in 1 L of distilled water [pH 6.1]), centrifuged as before, and the supernatant removed.

Naïve horses (numbers 1, 2, 5, 7, 9, 12, 13, 14, and 15) were inoculated IV with 20 μL of blood containing approximately 10^6 parasites directly from the splenectomized horse and diluted in 1 mL of physiological saline. Parasitemia was determined by quantitative real-time PCR as previously described (Ueti et al., 2008). Two horses (8 and 10) were not inoculated and served as negative controls. Inoculated animals were monitored clinically twice weekly for signs of disease such as pyrexia.

Detection of infection

To carry out nPCR, blood samples were collected in EDTA, and genomic DNA was extracted using a commercially available kit (Invimag Blood Mini Kit, Invitek). Samples of DNA were re-suspended in 100 μL of elution buffer and stored at 4 °C until required. The nPCR targeted *ema*-1, a single copy gene conserved among strains of *T. equi* (Knowles et al., 1992). Briefly, the 25 μL external reaction contained 0.4 μM each of external primers (forward: GAGGAGGAGAAACCCAAG and reverse: GCCATCGCCCTTGTAGAG), 12.5 μL of master mix (Roche), and 5 μL of the DNA sample. The external reaction was carried out in a thermal cycler with parameters as follows: 95 °C for 5 min followed by 30 cycles of 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s; and a final extension 72 °C for 5 min. The internal reaction was conducted in 25 μL containing 0.4 μM each internal primers (forward: TCAAGGACAACAAGCCATAC and reverse: TTGCCTGGAGCCTTGAAG), 12.5 μL master mix, and 1 μL of external reaction.

The nPCR internal reaction was carried out in a thermal cycler with parameters as follows: 95 °C for 5 min followed by 30 cycles of 95 °C for 5 s, 60 °C for 5 s, and 72 °C for 5 s; and a final extension of 72 °C for 5 min. The resulting amplicons were analyzed following 2% agarose gel electrophoresis. Horse β-actin nPCR was performed to determine the quality of DNA isolated (Schwint et al., 2008). To determine the threshold of detection of the nPCR, the assay was performed using serial dilutions of a plasmid containing an *ema-1* insert. We determined that this nPCR was capable of detecting <10 copies/μL (data not shown).

Serological assays were performed to confirm infection in the experimentally infected horses, and to generate a baseline to facilitate comparison of antibody levels following treatment. The CFT and indirect fluorescent antibody test (IFA) were performed according to the current World Organization for Animal Health (OIE) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Anonymous, 2008). The cELISA was performed using a commercially available kit (VMRD) according to the manufacturer's directions. Serum samples were tested in triplicate and the results reported as the mean percent inhibition (%I). According to the kit insert, results ⩾ 40% inhibition are considered positive.

Table 1
Identification and treatment details of horses used in the study.

Donor horse	Recipient horse
Treated	
1	404
5	406
7	403
13	407
14	405
15	408
Untreated	
2	8339
9	8345
12	409
Negative controls	
8	Not applicable
10	Not applicable

Administration of imidocarb dipropionate

Imidocarb dipropionate (Imizol, Schering Plough Animal Health) was administered as four sequential IM injections of 4 mg/kg bodyweight, 72 h apart. Treatment was initiated in horses 1, 5, 7, 13, 14, and 15 at 34, 36, 41, 27, 36, and 34 weeks post-inoculation (wpi), respectively. Simultaneous treatment of all horses in this group was not performed due to space constraints and in order to monitor horses for any adverse side effects. A second round of treatment as described above was given to horses 5 and 14 at 70 wpi.

Testing for elimination of T. equi by nPCR and blood sub-inoculation

Parasite clearance in the context of this experiment was arbitrarily defined as a negative nPCR result on three or more sequential bleeds. To confirm the negative nPCR results and to further determine the efficacy of imidocarb dipropionate, blood from all infected horses was sub-inoculated into naïve horses 9 months to 1 year following treatment. Sub-inoculation studies were staggered due to space constraints. Six horses (403, 404, 405, 406, 407 and 408) free of *T. equi* were used as recipients of blood from the treated horses (Table 1). Briefly, 500 mL of blood from each treated horse was collected in acid citrate dextrose solution. Following centrifugation at 900 *g* for 10 min, the buffy coat and plasma were removed, and the packed cells were washed once with Alsever's solution, centrifuged as before, and the supernatant decanted. The packed erythrocytes were re-suspended to 50% (v/v) in Alsever's solution. A total of 100 mL of blood was inoculated by jugular venipuncture into the naïve horses. Recipient animals were given blood from a single donor. In order to demonstrate this method as an effective mode of parasite transmission, the procedure was also performed using blood from three untreated controls (Nos. 2, 9, and 12). Three *T. equi*-free horses (Nos. 8339, 8345, and 409) were used as recipients (Table 1). All recipient horses were monitored for evidence of infection using nPCR, CFT, and cELISA.

Results

Acute disease in the experimentally infected horses was characterized by slight elevations in rectal temperature and a decreased hematocrit (data not shown). Overall, clinical signs were very mild, were not observed in all animals, and were no longer evident 3 months post-inoculation. During this acute phase, the time required to detect *T. equi* by the serological and molecular assays was compared (Table 2). Experimental infection was detected by nPCR approximately 1–2 wpi. Serology confirmed *T. equi* infection in all experimentally infected horses with specific antibodies first