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Research paper

Infection dynamics of *Theileria equi* and *Theileria haneyi*, a newly discovered apicomplexan of the horse

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ABSTRACT

Theileria equi infection, exotic to the United States has reemerged through intravenous (iatrogenic) and tick-borne transmission. Surveillance at the US-Mexico border identified a new species, *Theileria haneyi*, (*T. haneyi*^{EP}) (EP = Eagle Pass, Texas) which warranted additional investigation due to inability to detect by PCR targeting of *T. equi ema-1* and EMA-1-cELISA validated for *T. equi*. Infection dynamics of *T. haneyi*^{EP} were evaluated, including ability to superinfect in the presence of *T. equi*-Texas (*T. equi*^{TX}), the isolate responsible for the re-emergence of *T. equi* in the U S. Experimental infection with *T. equi*^{TX} or *T. haneyi*^{EP} revealed minimal clinical disease however, *T. equi*^{TX} infection led to significantly greater neutropenia. Comparison of time to antibody detection following inoculation revealed significantly greater time to detectable anti-*T. haneyi*^{EP} antibody (26.67 days post-inoculation (DPI)) than *T. equi*^{TX} (11.67 DPI). Regardless of initial infection with either *T. equi*^{TX} or *T. haneyi*^{EP}, superinfection was established. Comparative analysis of antibody responses from a splenectomized horse infected with *T. haneyi*^{EP} to that of a spleen intact horse infected with *T. equi*^{FL} revealed a different antibody binding profile to *T. haneyi*^{EP}, *T. equi*^{TX} and *T. equi*^{FL} merozoite antigen and limited shared antigen/cross-reactive antibody(s). Affinity purified *T. equi* EMA-1 and EMA-2 from *T. equi*^{FL} were shown as targets for horse antibodies against *T. haneyi*. Data presented here show (1) *T. haneyi*^{EP} can superinfect in the presence of *T. equi*^{TX} infection and co-persists for minimally 25 months, (2) intravenous challenge with *T. haneyi* is subclinical, and (3) limited cross-reactive antibody between *T. haneyi*^{EP} and *T. equi* includes reactivity to EMA-1 and EMA-2.

1. Introduction

Equine piroplasmosis is caused by *Theileria equi* (*T. equi*) and *Babesia caballi* (*B. caballi*). Recently, *T. equi* infection reemerged through both iatrogenic and tick-borne transmission in the United States (Beard et al., 2013; Scoles et al., 2011; Short et al., 2012; Wise et al., 2013). As is the case for other Apicomplexan diseases such as malaria and babesiosis, immune prophylaxis other than premonition is not available. In contrast to much of North America, these tick-borne apicomplexan parasites are endemic throughout tropical and subtropical regions of the world (Wise et al., 2013). Infection by *T. equi* classically causes non-specific clinical disease, characterized by fever, malaise, and signs associated with hemolytic anemia of variable severity. Clinical severity is hypothesized to be the culmination of multiple factors including parasite strain, horse immune and nutritional status, infected tick density and the infective dose; with the threshold of these variables unknown at this time (Wise et al., 2013). Horses that survive acute infection subsequently become persistently-infected, asymptomatic carriers for life and are reservoirs of infectious organisms (Ueti et al., 2008). The asymptomatic carrier is a significant burden on the international movement of horses in that *T. equi* infected horses are restricted from entering several countries including the US.

The recent discovery of *T. equi* infection in Texas exemplifies a common clinical presentation in non-endemic countries with the vast majority of infections being asymptomatic (Wise et al., 2013). Transmission resulting in 297 affected horses was shown to be due, at least in

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part, to a newly recognized competent tick vector in the region, *Amblyomma mixtum*, formally known as *Amblyomma cajenneense* (Scoles et al., 2011). Additional infections in Florida and Missouri, however, were caused by iatrogenic transmission and resulted in more clinically apparent disease in some horses (Beard et al., 2013; Short et al., 2012). These outbreaks resulted in intensified surveillance and enabled the identification of a new species, *Theileria haneyi,* in the horse (Knowles et al., 2018). The organism was initially suspected to be a new *T. equi* isolate; however, when infection was not detectable by methods validated for *T. equi*, further genomic evaluation was pursued (Knowles et al., 2018). Genome diversity and 18S phylogenetic analysis led to definition of the new species. Absence of detectability by serologic testing or PCR using *T. equi* Equine Merozoite Antigen-1 (EMA-1) or (*ema-1*gene sequences was demonstrated to be the result of changes within the *ema* superfamily between *T. equi* and *T. haneyi* (Knowles et al., 1991, 2018). Fortunately, EMA-1 and EMA-2 share an epitope as defined by mAb 36/133.97, and although antigenic cross-reactivity among the EMAs is assumed, additional shared epitopes have not been defined. (Knowles, et al., 2018).

A recent study of horses persistently infected with *T. equi* showed that when parasite genotype is defined by microsatellite DNA sequence, 27% of infections contained multiple isolates (Hall et al., 2013). Natural, mixed infections with *T. equi* and *B. caballi* have been reported and reproduced experimentally (de Waal et al., 1987), but examination of superinfection or co-infection with another *Theileria* species has not been warranted in the horse until the recent discovery of *T. haneyi*^{EP} (Knowles et al., 2018).

Infections with other apicomplexans, such as *Plasmodium* spp., commonly involve more than one genotype or species in endemic areas, yet individuals with mixed malarial infections are also more likely to be asymptomatic (Fairlie-Clarke et al., 2013; Sitali et al., 2015). Superinfection in malaria can be inhibited by circulating parasitemia in a density-dependent manner (Portugal et al., 2011). Additionally, inoculation of sporozoites of one *Plasmodium* spp. has been shown to provide significant cross-protection against other *Plasmodium* spp. (Sedegah et al., 2007). Other studies in Apicomplexans have also shown variable levels of cross-species/isolate protection are afforded following infection (Atkinson et al., 2001; Benach et al., 1979; Sedegah et al., 1982; Wright et al., 1987). Therefore, further exploration of superinfection dynamics with *T. equi* and the novel, closely related species, *T. haneyi* would be valuable especially considering the recent identification of the diagnostically evasive species, *T. haneyi* within the US and discovery of multiple naturally infected cases (Knowles et al., 2018).

Therefore, the aim of this study was to evaluate both primary and superinfection dynamics for *T. haneyi*^{EP} and *T. equi*^{TX} including evaluation of parasitemia (time to development and peak during primary infection), changes in physical examination and hematologic parameters, and time to development of a humoral response by immunoblot and cELISA. The *T. equi*^{TX} isolate was chosen due to the geographic proximity of the site of discovery of *T. haneyi*^{EP} to the recent *T.equi*^{TX} outbreak in South Texas (Scoles et al., 2011; Knowles et al., 2018).

2. Materials and methods

2.1. Animal selection

Seven healthy, mixed-breed yearlings of both sexes (4 intact males & 3 females) from the USDA-ARS research herd were used (Moscow, Idaho, USA). Eligibility for the study required a normal initial complete physical examination, normal hemogram and biochemical profile, and a negative result on nested PCR (nPCR) for both *B. caballi* and *T. equi*. Six of the seven yearlings were randomly assigned to one of two groups (*T. equi*^{TX} or *T. haneyi*^{EP}) with both groups consisting of 2 males and 1 female. All horses were housed in 12 × 12 stalls with the two fillies being stalled together, and the four colts being separated into 2 groups of 2. Horse 301 (Ho-301) was splenectomized and housed in a separated stall alone. Serum from an additional horse, Horse 5 (Ho-5), that was intravenously infected with *T. equi*^{FL} as previously described (Knowles et. al., 1991), was also used in the study. The horses were transitioned from their previous pasture housing to stalls 3 weeks prior to experimental inoculation, and daily handling sessions were initiated to prevent artificial elevations in physical examination and hemogram parameters. All experimental procedures were approved by the University of Idaho, Institutional Animal Care and Use and Biosafety Committees (Protocol Numbers, IACUC: 2013-66, Biosafety: B-010-13) in accordance with institutional guidelines based on the U.S. National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

2.2. Animal inoculation

The three horses in the *T. equi*^{TX} group (Ho-275, Ho-277, Ho-278) were inoculated intravenously with a *T. equi*^{TX} stabilate (38% percent parasitized erythrocytes (PPE)) diluted in 10% normal horse serum in phosphate buffered saline (PBS). Each of the three horses in the *T. haneyi*^{EP} group (Ho-273, Ho-280 and Ho-283) was inoculated intravenously with a *T. haneyi* stabilate (12% PPE) diluted with normal horse serum in phosphate buffered saline (PBS). In order to equilibrate the percentage of parasitized erythrocytes given to all six horses, the *T. equi*^{TX} stabilate was divided into equal one-milliliter aliquots between the three horses in the *T. equi*^{TX} group. The splenectomized horse (Ho-301) was inoculated with 120 ml of whole blood from a confirmed persistently infected *T. haneyi*^{EP} *horse* (Knowles et.al., 2018). Persistent phase of infection is defined as resolution of anemia (when present), parasitemia at ≤ 0.001 infected erythrocytes and serological and nPCR positivity.

Twelve weeks after initial inoculation, and horses were demonstrated to be in persistent phase of infection by serology and nPCR, secondary infection (superinfection) was attempted with two horses (Ho-275 and Ho-278) from the *T. equi*^{TX} group and two horses (Ho-280 and Ho-283) from the *T. haneyi* group. The *T. equi*^{TX} horses were each inoculated with *T. haneyi* stabilate (12% PPE), and the *T. haneyi* horses were each inoculated with one-milliliter aliquots of a *T. equi*^{TX} stabilate (38% PPE). Secondary infection was attempted a second time 20 months after initial inoculation in the one horse that had failed to become superinfected on initial attempt (Ho-280) and in the two horses that had remained singly infected (Ho-273 and Ho-277). For the purpose of this study, superinfection is defined as secondary infection of a horse with either *T.equi*^{TX} or *T. haneyi*^{EP} that is previously confirmed to be infected following primary inoculation with either *T. equi*^{TX} or *T. haneyi*^{EP}. Superinfection is considered established when a horse becomes and remains PCR positive for both the primary and secondary infecting species.

2.3. Clinical evaluation of infected horses

Daily physical examinations were performed on all 6 horses assigned to the *T. equi*^{TX} and *T. haneyi* groups for 6 weeks post-primary inoculation. Heart rate (< 48 beats per minute), respiratory rate (< 20 breaths per minute), rectal temperature (< 101.0 °F), mucous membrane color and character, capillary refill time, mentation, and presence of gastrointestinal borborygmi were evaluated. Whole blood and serum samples were collected by jugular venipuncture daily for the first 4 weeks, then every other day for an additional 2 weeks, followed by weekly for an additional 6 weeks. Packed cell volumes (PCV) were measured daily for the first 28 days and hemograms were evaluated 3 days per week for the first 6 weeks. Hemograms were then evaluated weekly for an additional 3 weeks. After secondary inoculation, all four horses (Ho-275, Ho-278, Ho-280 and Ho-283) were evaluated daily for the first 30 days. PCVs were measured daily for the first 28 days and hemograms were evaluated 3 days per week for the first 6 weeks and then weekly for an additional 2 weeks. Following inoculation of the

remaining, singly infected horses, monitoring was limited to daily rectal temperatures and general observation of their attitudes and appetite, blood draws were also reduced to 14-day intervals. Monitoring of the splenectomized horse was limited to daily rectal temperature, PPE, and PCVs as well as general observation of her attitude and appetite.

Blood smears were made the day of sample collection and stained with Diff-Quik®. Percent-parasitized erythrocytes (PPE) were determined by microscopic evaluation of stained smears on the following days for spleen-intact horses (0–10, 12–16, 18, 21, 23, 29–30). For the splenectomized horse, blood smears were evaluated daily for the first 9 weeks post-inoculation and then weekly for the subsequent 10 weeks. Whole blood collected in EDTA was centrifuged at 1250 x g for 10 min, and packed red blood cells (RBCs) were then aspirated and stored at −20 °C prior to DNA isolation. Serum was also stored at −20 °C. Due to the challenge of identifying *T. equi* and *T. haneyi* by light microscopy at low parasitemia, nPCR was also used to show infection and superinfection.

2.4. Nested PCR for T. equi and T. haneyi detection of single and superinfected horses

Genomic DNA was isolated from packed red blood cells as previously described (Ueti et al., 2012). nPCR for *T. equi*^{TX} utilized primers for the *ema-1* gene that was found to be absent on genomic analysis of *T. haneyi*^{EP} (Knowles et al., 2018; Ueti et al., 2008). Naïve horses were evaluated on the following days post- primary inoculation: 6, 10, 15, and 21 in order to confirm infection with either *T. equi*^{TX} or *T. haneyi*^{EP}. For confirmation of superinfection, horses were evaluated first on day 15 and then evaluated at 7 to14-day intervals until the horses were confirmed positive for the superinfecting species. Infection status was then monitored periodically, thereafter, over the subsequent 42 months.

2.4.1. Theileria haneyi nested PCR

Genomic sequencing and comparative analysis between *T. haneyi*^{EP} and *T. equi* enabled the identification of the extensive changes that had occurred to *ema* family members on chromosome 1, including the absence of *ema* 1, 3, and 4 as compared with *T. equi*^{FL} (Knowles, et al., 2018). The recombination event that led to the absence of an *ema1* (BEWA_026850) paralog also affected other genes in the syntenous locus of *T. haneyi*^{EP}, including the addition of a unique 2118 base pair open reading frame within base range 1747400–1749517 of chromosome 1. Indications are that this is a single copy gene by comparison to the *T. haneyi*^{EP} nucleic acid sequence (Knowles et al., 2018). Primers were designed for nPCR with the following sequences: ExtFor 5′ CCA TACAACCCACTAGAG 3′, ExtRev 5′ CTGTCATTTGGGTTTGATAG 3′, IntFor 5′ GACAACAGAGAGGTGATT 3′, and IntRev 5′ CGTTGAATGTA ATGGGAAC 3′ resulting in a 238 bp product. Thermal cycling conditions for external primers are 95 °C for 4 min, followed by 35 cycles of 95 °C for 20 s, 63.5 °C for 30 s and 72 °C for 20 s, and then 72 °C for 7 min extension. The cycling conditions for the internal primers are the same except for a 58.1 °C annealing temperature in place of 63.5 °C. External primers were used at 2.5 μM per primer final concentration and internal primers were used at 7.5 μM final concentration. PCR Master Mix (Roche, Indianapolis, IN, USA) was used in a total 25 μL reaction, beginning with 5 μL isolated DNA in the external reaction, and 1 μL of external reaction used as template in the internal reaction.

2.5. Detection of T. equi specific serum antibodies

The World Organization for Animal Health (OIE) and the United States Department of Agriculture (USDA) approved regulatory diagnostic test, a competitive, enzyme-linked, immunosorbent assay (cELISA) for *T. equi*, was performed using a commercially available kit as directed by the manufacturer (VMRD, Pullman, WA, USA). Horses were tested serially over the course of both primary and secondary infection. A positive result is defined as > 40% inhibition by the manufacturer.

2.6. Immunoblotting with whole T. equi and T. haneyi merozoite antigens

Whole merozoite antigen was isolated and prepared as previously described for Ho-196, a horse that was infected with *T. equi*^{TX}, and Ho-208, a splenectomized horse infected with *T. haneyi*^{EP} (Silva et al., 2013). Lysates of each species were separated on NuPAGE Novex 4–12% Bis-Tris protein gels with MOPS running buffer, and separated proteins transferred to nitrocellulose using NuPAGE transfer buffer (Invitrogen NP0006, Thermo Fisher Scientific, Waltham, MA) containing 10% methanol. Antibody reactivity was evaluated using pre- and post-inoculation sera as previously described (Silva et al., 2013). Pre-inoculation sera from all 6 horses and then post-inoculation sera following primary infection with either *T. haneyi*^{EP} or *T. equi*^{TX} from each horse at 4-day intervals were evaluated to determine time to development of an antibody response. To evaluate time to development of humoral response during superinfection, sera from each superinfected horse was evaluated with the secondarily inoculated antigen at 7-day intervals during the initial superinfection attempt. Sampling was less aggressive upon second inoculation of the remaining 3 horses (Ho-273, Ho-277, Ho-280), and all 3 horses were evaluated at approximately 7 to14-day intervals. Additionally, serum from a horse inoculated with *T. equi*^{FL} (H5), a horse utilized in previous studies (Knowles et al., 1991), was evaluated with whole merozoite antigen from *T. haneyi*^{EP}, *T. equi*^{FL}, and *T. equi*^{TX} to determine any cross-reactivity between the well-characterized *T.equi*^{FL} strain and *T. haneyi* (Knowles et al., 1991).

2.7. Immunoblotting with immunopurified T. equi^{FL} EMA-1/2 antigen

Immunoblot antigen was made from fresh, defibrinated blood from *T. equi*^{FL} infected horses as previously described (Silva et al., 2013). Lysate was then further purified as previously described utilizing monoclonal antibody 36/133.97, allowing for elution from an affinity column of immunopurified EMA-1/2 (Kappmeyer et al., 1993). The immunopurified lysate was mixed with NuPAGE 4X LDS sample buffer and 10 X reducing buffer (Thermo Fisher Scientific, Waltham, MA) and boiled for 5 min. Samples were electrophoresed on NuPAGE Novex 4–12% Bis-Tris protein gels with MOPS running buffer, and separated proteins were transferred to nitrocellulose using NuPAGE transfer buffer (Thermo Fisher Scientific) containing 10% methanol. The membrane was blocked in dilution buffer (10% nonfat dry milk) at 4 °C overnight. The membrane was cut into strips and incubated individually with pre- and post-inoculation sera from Ho-301 or monoclonal antibodies in dilution buffer. Horse sera was diluted to 1:100, and mAb concentration was 5 μg/mL. Following washing in PBS/0.2% Tween 20, secondary antibody conjugated with horseradish peroxidase anti-horse or anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at 1:10,000 in blocking solution was used. After washing in PBS/ 0.2% Tween20, membranes were incubated with ECL reagent (GE Healthcare Biosciences, Pittsburgh, PA), and film exposed.

2.8. Statistical analysis

An unpaired, two-tailed student *t*-test was utilized to assess differences between the *T. haneyi* primary infection, *T. equi* primary infection, *T. equi* secondary infection, and *T. haneyi* secondary infection groups.

3. Results

3.1. Establishment of primary infection and superinfection with T. equi^{TX} and T. haneyi^{EP}

As multiple recent outbreaks have been associated with iatrogenic

Table 1
Disease parameters after primary inoculation with *T. equi*^{TX} (TX) or *T. haneyi*^{EP} (TH).

Horse	Isolate	Peak PPE - DPI		Duration of anemia (days)	Lowest PCV - DPI		Lowest Neutrophil count (x10^3 cells /μL) - DPI	
Ho-275	TX	0.03%	13	29	21%	22	0.039	36
Ho-277	TX	0.05%	12	3	26%	16	0.348	36
Ho-278	TX	0.40%	18	43	17%	15	0.616	36
Ho-273	TH	None seen	–	2	29%	22	1.54	39
Ho-280	TH	0.03%	23	19	25%	30	1.98	28
Ho-283	TH	0.02%	13	15	26%	30	2.70	19

PPE: Percent of Parasitized Erythrocyte; DPI: days post-inoculation; PCV: Packed Cell Volume; Lowest neutrophil count: lowest recorded value during the 56-day sampling period. Values are reported with corresponding DPI of recorded value. Hemograms were evaluated three days per week during the 8-week sampling period, and PCVs were evaluated 5 days per week during the first 30 days. The normal neutrophil count range in adult horses is 3.0–7.0 × 10^3 cells/μL (SI Unit equivalent: x10^9 cells/L).

Table 2
Time to development of detectable parasitemia by nPCR, humoral response by immunoblot and EMA1-cELISA after primary inoculation with *T. equi*^{TX} (TX) or *T. haneyi*^{EP} (TH).

Horse	Species	nPCR (dpi)		Immunoblot (dpi)		EMA-1 cELISA (dpi)
		T. equi	*T. haneyi*	*T. equi* Ag	*T. haneyi* Ag	
Ho-275	TX	15	–	17	–	53
Ho-277	TX	10	–	11	–	42
Ho-278	TX	10	–	7	–	36
Ho-273	TH	–	10	–	25	N
Ho-280	TH	–	10	–	30	N
Ho-283	TH	–	10	–	25	N

Time is reported as days post-inoculation (dpi). Time to confirmation of infection by nested PCR is also reported.

transmission, intravenous inoculation in naïve, spleen-intact horses with either *T. haneyi*^{EP} or *T. equi*^{TX} was evaluated. Following intravenous inoculation with equivalent doses of *T. haneyi*^{EP} or *T. equi*^{TX} infected erythrocytes, both groups of 3 horses were confirmed to be infected by species-specific nPCR (Table 2). Additionally, serial blood smears were also evaluated to further confirm infection. Parasites were more commonly seen during primary infection with *T. equi* between days 8 and 18 post-inoculation with peak parasitemia of 0.40% observed on day 18 (peak parasitemia range: 0.03-0.40%). With *T. haneyi* primary infection, intracellular organisms were initially observed slightly later than for *T. equi*, on day 13 with intermittent detection thereafter. One of the three *T. haneyi* infected horses showed peak parasitemias of only 0.03% on day 23 (peak parasitemia range: 0–0.03%) (Table 1). These differences in the aforementioned parasitemia dynamics between *T. equi* and *T. haneyi* infected groups were not significant.

Twelve weeks after initial inoculation, two of the three *T. equi*^{TX} persistently infected horses (Ho-275 and Ho-278) were secondarily inoculated with *T. haneyi*^{EP} *and* were both confirmed to be superinfected by nPCR (Table 3). Superinfection was later attempted in the remaining *T. equi*^{TX} persistently infected horse (Ho-277) and was also confirmed to be superinfected with both species by nPCR (Table 3). The horses initially shown to be superinfected (Ho-275 and Ho-278) were evaluated 42 months after inoculation and remained persistently superinfected with both *T. equi*^{TX} and *T. haneyi*^{EP}. Horse (Ho-277) that was secondarily inoculated at a later time point was also confirmed to be persistently superinfected with both *T. equi*^{TX} and *T. haneyi* 25 months post-inoculation.

Superinfection of *T. haneyi* persistently infected horses with *T. equi*^{TX} was also attempted. Two of the three *T. haneyi* persistently infected horses (Ho-280 and Ho-283) were secondarily inoculated with *T. equi*^{TX}. After secondary inoculation with *T. equi*^{TX}, only Ho-283 was confirmed to be superinfected by nPCR. Superinfection as defined by nPCR was not established in Ho-280. Superinfection was later attempted in the remaining *T. haneyi* persistently infected horse (Ho-273) and attempted a second time in Ho-280. Both horses were confirmed to be superinfected by nPCR (Table 3). The horse initially shown to be superinfected (Ho-283) was evaluated 42 months after inoculation and remained persistently superinfected with both *T. equi*^{TX} and *T. haneyi*. The 2 other horses (Ho-273 and Ho-280) that were superinfected at a later time point were also confirmed to be persistently infected with both *T. equi*^{TX} and *T. haneyi* 25 months post-inoculation.

3.2. Infection and superinfection by T. haneyi are clinically inapparent

In conjunction with evaluation of infection dynamics, we concurrently evaluated the clinical course of both single and superinfection. Overall, only mild changes in physical examination parameters were observed with only one horse in each group developing a mild fever (101.1–103.2 °F) of 48–72 hours duration.

Serial hemograms showed predominantly mild changes for both groups. Primary infection with *T. equi* resulted in mild to moderate anemia as compared to primary infection with *T. haneyi*. The lowest recorded PCV value following primary inoculation for the *T. equi* group was 17%, and for the *T. haneyi* group was 25%, but there was no significant difference between the two groups, $p = 0.1364$ (Table 1). Comparable declines were also seen in both erythrocyte counts and hemoglobin concentrations. Duration of anemia for primary infection with either group was variable, ranging from 3 to 43 days for *T. equi*^{TX} and 2 to 19 days for *T. haneyi* (Table 1). All 3 horses in the *T. equi*^{TX} group became markedly neutropenic with all declining to less than a 1000 cells/uL between days 30 and 36 with the lowest measured values in all 3 horses occurring on day 36 (Table 1). In comparison, the *T. haneyi* group became less neutropenic with one horse developing a neutrophilia. Ho-283 developed a mature neutrophilia from day 25 to 36 that peaked at 11,115 cells/uL with mild toxic changes the day he was moderately febrile. No immature neutrophils were observed. Overall, no significant difference in hematologic parameters were found except for the degree of neutropenia, as the *T. equi*^{TX} primary group developed a significantly greater neutropenia as compared with horses initially infected with *T. haneyi*, $p = 0.005$ (Table 1).

Following the initial attempt of superinfection, all 4 horses (Ho-275, Ho-278, Ho-280 and Ho-283) were evaluated daily for any changes in their physical examination parameters. All four horses only showed mild changes in their physical examination parameters regardless of the superinfecting species. Serial evaluations of hemograms were again performed and revealed minimal changes in PCV, hemoglobin, and erythrocyte counts. Neutrophil counts fluctuated from mild to moderately low to within the reference range over the 8-week evaluation period. Greater declines were observed in *T. equi*^{TX} horses superinfected with *T. haneyi*^{EP} (Ho-275 and Ho-278) with the lowest values recorded on day 38 post-super inoculation (Ho-278 with 900 cells/uL) and day 47 (Ho-275 with 732/uL). The lowest recorded value for the *T. haneyi* horse (Ho-283) that was successfully superinfected with *T. equi*^{TX} was on day 3 (2,160 cells/uL).

3.3. Establishment of persistent T. haneyi infection in a splenectomized horse

A splenectomized horse (Ho-301) was inoculated with *T. haneyi* to test the hypothesis of decreased virulence. During the 10-week observation period, blood smears and PCVs were evaluated daily. Parasites were initially visible 7 days post-inoculation with the peak PPE of 1.08% observed on day 30. Parasites were consistently observed

Table 3
Time to development of detectable parasitemia by nPCR, anti-*T. equi* antibody response by immunoblot and EMA1-cELISA after superinfection with either *T. equi*^{TX} (TX) or *T. haneyi*^{EP} (TH).

Horse	Initial species	Secondary species	nPCR(dpi)		Immunoblot (dpi)		EMA-1 cELISA (dpi)
			T.equi	*T. haneyi*	*T. equi* Ag	*T.haneyi* Ag	
Ho-275	TX	TH		21	**	Not determined	**
Ho-277	TX	TH		28	**	28	**
Ho-278	TX	TH		15	**	35	**
Ho-273	TH	TX	28		28	–	42
Ho-280[1]	TH	TX	28		56	–	15
Ho-283	TH	TX	56		70	–	84

**All horses initially infected with *T.equi*^{TX} remained positive at all evaluated time points.
[1] Superinfection confirmed by nPCR after repeated inoculation with *T.equi*^{TX} on second occasion. Time to confirmation of superinfection by nested PCR is also reported. Time is reported as days post- secondary inoculation (dpi).

from day 7 to 60. The PCV gradually declined with the lowest value (23%) recorded on day 51 post-inoculation. Ho-301 developed a mild fever on day 13 and then a more significant fever of 48 h duration on day 51 that resolved without further treatment. Ho-301 remains persistently infected 36 months post-inoculation as defined by blood smear, nPCR, and immunoblot.

3.4. EMA family cross-reactivity between *T. equi*^{FL}, *T. equi*^{TX}, and *T. haneyi*^{EP}

Antigenic cross-reactivity was observed in one-dimensional immunoblot between sera from a *T. equi*^{FL} infected horse (H5) and *T. equi*^{TX} whole merozoite antigen (Fig. 1). However, antigenic cross-reactivity using anti-*T. equi*^{FL} serum (H5) against *T. haneyi*^{EP} antigen was limited to an immunoreactive band at approximately 29 kDa (Fig. 1). Serum from a long-term *T. haneyi*^{EP} persistently infected, spleen-intact horse (Ho-273) yielded a band corresponding to the molecular mass of EMA-2 when tested against *T. equi*^{TX} (data not shown). To further define this antigenic cross-reactivity between *T. haneyi* and *T. equi*, sera from *T. haneyi* infected, splenectomized horse (Ho-301) was evaluated by immunoblot with immunoaffinity purified *T. equi*^{FL} EMA-1/-2 antigens (Fig. 2). Serum from *T. haneyi*^{EP} infected, splenectomized horse (Ho-301) bound immunoaffinity purified EMA-1 and EMA-2 (Fig. 2). Additional binding at approximately 50 kDa was also present in lanes 2, 3 and 4 of Fig. 2, including the isotype control monoclonal antibody

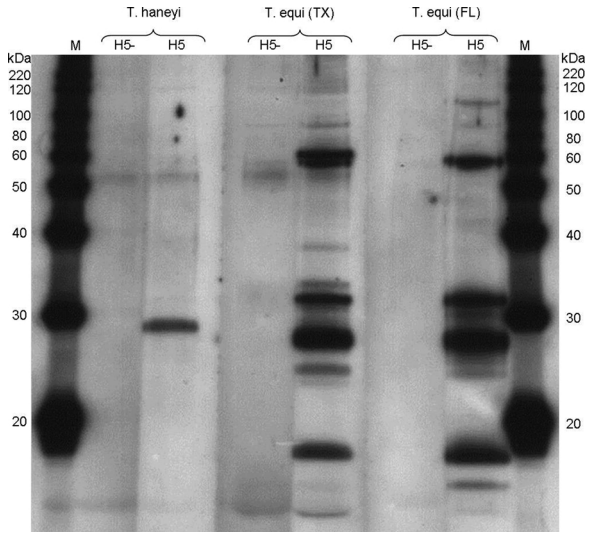

Fig. 1. Immunoblot showing reactivity of serum from *T. equi*^{FL} infected horse (H5) with *T. haneyi*^{EP} (TH), *T.equi*^{TX} (TX), and *T.equi*^{FL} (FL) whole merozoite antigens. H5- and H5+ denotes pre-inoculation and post-inoculation sera respectively. All samples were diluted to 2×10^{-3}.

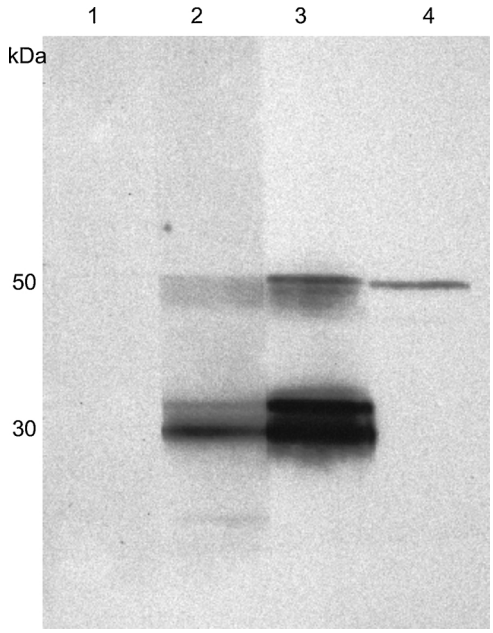

Fig. 2. Immunoblot showing reactivity to mAb 36/133.97 affinity purified native *T. equi*^{FL} EMA1 and EMA2. mAb 18.185 raised against *Cryptosporidium parvum* is used as an IgG1 isotype control. Molecular weight indications are derived from Invitrogen Novex Sharp Prestained protein standard. Shown are pre-inoculation sera, Ho-301 (lane 1), post-inoculation sera, Ho-301 (lane 2), mAb 36/133.97 (lane 3), mAb 18.185 isotype control (lane 4).

(mAb). The binding at 50 kDa by post-infection serum from Ho-301 and mAb36/133.97 are likely dimers of EMA-1/2. The molecular mass of the immunoreactive band in lane 4, bound by the isotype mAb is slightly less than that in lanes 2 and 3.

3.5. Time to development of antibody responses during superinfection

Following primary and secondary inoculations, time to development of a detectable antibody response was evaluated through both immunoblot and cELISA. During primary inoculation, horses infected with *T. equi*^{TX} (Ho-275, Ho-277, Ho-278) became positive by cELISA 36 to 53 DPI, and horses infected with *T. haneyi*^{EP} (Ho-273, Ho-280, Ho-283) were negative, which is consistent with previous data (Table 2) (Knowles et al., 2018). Sera from all horses secondarily inoculated with *T. haneyi*^{EP} (Ho-275, Ho-277, and Ho-278) remained positive by cELISA with > 40% inhibition recorded at all time points. Horses confirmed to be secondarily infected with *T. equi*^{TX} (Ho-273, Ho-280, Ho-283) did become positive by cELISA. Ho-283 became positive on day 84 and Ho-273 on day 42 post-inoculation. Ho-280 was negative throughout the course of the initial superinfection evaluation period due to failure to

establish secondary infection with *T. equi*^{TX}. However, upon a second superinfection attempt, Ho-280 became positive 15 days after challenge with *T. equi*^{TX} (Table 3). There was no significant difference in time to cELISA positive between horses primarily inoculated with *T. equi*^{TX} and horses secondarily inoculated with *T. equi*^{TX}, p = 0.8798.

Immunoblot analysis was subsequently utilized to assess the time to detection of antibody responses during both primary and superinfection. The time to development of a humoral response during primary infection was defined as the timeframe from inoculation to initial detection of at least one immunoreactive band that correlated to a band of similar molecular mass of the inoculating species' positive control. The mean time to initial detection of an antibody response to primary infection with *T. equi*^{TX} was 11.67 DPI, and the mean time to detection of an antibody response to primary infection with *T. haneyi*^{EP} was 26.67 DPI, which was significantly greater as compared with the mean time to detection for *T. equi*^{TX} horses, p = 0.0055.

During superinfection, time to development of a humoral response was defined as the time at which a band of greater density as compared with pre-inoculation samples was identified for persistently infected *T. haneyi* horses secondarily inoculated with *T. equi*^{TX} (Ho-273, Ho-280, Ho-283). The use of band density to detect superinfection was necessary due to the cross reactivity at the molecular mass of EMA-2 as previously defined. For persistently infected *T. equi*^{TX} horses secondarily inoculated with *T. haneyi*^{EP} (Ho-275, Ho-277, Ho-278), time to development of a humoral response was defined as the time to development of a second band at approximately 16 kDa which is unique to *T. haneyi*^{EP} infection. Therefore, during superinfection, time to detection of an antibody response to secondary infection with *T. haneyi*^{EP} (Ho-275, Ho-277, Ho-278) and time to detection of an antibody response to secondary infection with *T. equi*^{TX} (Ho-273, Ho-280, Ho-283) was 31.5 DPI and 51.3 DPI, respectively. The calculation of the average for horses secondarily inoculated with *T. haneyi* only includes two horses, as Ho-275 never formed a second band during the superinfection-sampling period (Table 3). When comparing time to detection of an antibody response to primary inoculation with *T. equi* and to secondary inoculation with *T. equi* following establishment of persistent infection with *T. haneyi*, time to detection was significantly prolonged, p = 0.01764.

4. Discussion

Data presented in this study show that *T. haneyi* is capable of establishing a clinically silent, persistent infection in naïve horses. Despite the limited antigenic cross-reactivity with *T. equi* and a possible comparative reduction in virulence, *T. haneyi* was also able to establish superinfection in the presence of anti-*T.equi* immunity, and ultimately long-term co-persistence with *T. equi*. Further evaluation of antigenic cross-reactivity revealed anti-*T. haneyi* antibody bound the highly conserved *T. equi*^{FL} EMA-1 and EMA-2 and potentially other EMAs at the 29 kDa molecular mass (Knowles et al., 1991). However, the antigenic cross-reactivity between *T. haneyi* and *T. equi* didn't prohibit the establishment of superinfection by either organism or subsequent co-persistence for 25 months. Therefore, the stealth emergence and persistence of *T. haneyi* raises concerns of its global prevalence and eventual impact on movement of equids as it is undetectable clinically or with *T. equi* specific diagnostics.

These data show that iatrogenic transmission, specifically intravenous inoculation to naïve horses, is an efficient route for the establishment of *Theileria* spp. infection in the horse supporting its epidemiological importance. Previous case reports have strongly suspected that intravenous inoculation through needle sharing or blood doping was the likely means of transmitting the parasite as well as previous experimental studies utilizing intravenous inoculation with other isolates (Beard et al., 2013; Kuttler et al., 1986; Short et al., 2012; Tenter and Friedhoff, 1986). However, there are no reports until now evaluating this route for either primary infection with *T. equi*^{TX} or *T. haneyi* or for establishment of superinfection with two *Theileria* spp. in the horse.

Experimental horses in this study regardless of the primary inoculating species were confirmed to be superinfected with both *T. haneyi* and *T. equi*^{TX}. The first attempt at superinfection with *T. equi*^{TX} resulted in two of the three *T. haneyi* horses becoming infected with *T. equi*^{TX} (Ho-283 and Ho-273). However, when secondary inoculation with *T. equi*^{TX} was repeated for Ho-280, superinfection with *T. equi*^{TX} was established. Ho-280 developed a positive anti-*T. equi* cELISA response by 15 dpi. The reduced time to a positive cELISA by Ho-280 compared with Ho-273 and Ho-283 (42 & 84 dpi to cELISA positive, respectively) is hypothesized to be the result of immune priming by the first superinfection attempt. Failure to establish superinfection in Ho-280 may have been due to error during preparation or handling of the stabilate or immunity induced by *T. haneyi* inhibiting infection by *T. equi*^{TX}. Ho-283, inoculated with an aliquot from the same *T.equi*^{TX} stabilate became superinfected and superinfection was confirmed at multiple time points. However, Ho-283 did show a marked delay in time to establishment of a measurable humoral response to *T.equi*^{TX} (70 dpi). With the same relative *T. equi* dose using a different stabilate, superinfection in Ho-273 was established on the first attempt.

Within the limited number of horses tested here, it appeared primary infection with *T. haneyi* delayed time to detection of an anti-*T. equi*^{TX} antibody response. Yet, primary *T. equi*^{TX} infection did not delay time to detection of anti-*T. haneyi* antibody. Evaluation of antigenic cross-reactivity revealed anti-*T. haneyi* antibody bound the highly conserved *T. equi*^{FL} EMA-1 and EMA-2 and potentially other EMAs at the 29 kDa molecular mass (Knowles et al., 1991). Similarly, in Fig. 1, anti-*T. equi* antibody from H5 bound an approximately 29 kDA protein suggesting that the antigenic cross-reactivity is at least in part within the EMA family. Immunodominant, shared antigens have been in turn implicated in eliciting an immune response capable of interspecies inhibition through evaluation of sequential infection with different *Plasmodium* and *Babesia* species (Cox, 1970; Ray et al., 1994; Terkawi et al., 2007). Previous work has shown that there is significant conservation of the EMA family between the two *Theileria* species and the molecular weight of the immunodominant band corresponds to that of multiple EMA family members (Knowles et al., 2018). The limited nature of the cross-reactivity provided by immunoblot analysis may provide insight as to which conserved antigens could elicit a protective response, as molecules identified by cross-reactivity can induce significant cross-protective immunity between species (Terkawi et al., 2007). Additionally, some of the binding at 50 kDa by the isotype control mAb, and mAb36/133.97 may be due to mouse immunoglobulin heavy chains eluted off of the affinity column with subsequent detection by the anti-mouse secondary antibody used against the mAbs including the isotype control. However, further investigation would be warranted to define the role that cross-reactivity may play in delaying establishment of infection.

Overall, the clinical picture elicited by iatrogenic transmission of *T. haneyi*^{EP} was suggestive that the parasite could be less virulent than other previously reported isolates of *T. equi*. No significant difference in PCV was found between the two groups. However, *T. equi*^{TX} infected horses did develop a significantly lower neutrophil count as compared with horses infected with *T. haneyi*^{EP}. Previous studies evaluating hematologic parameters have not evaluated or found a significant neutropenia (Kuttler et al., 1986; Singh et al., 1980; Zobba et al., 2008). However, neutropenia was mentioned to be associated with acute infection by De Waal et al in his 1992 review of equine piroplasmosis but further characterization was not mentioned nor has it been described during experimental infection (de Waal, 1992). However, in malaria, specifically in acute infection with *P. vivax,* neutropenia is common but is typically characterized by increased numbers of non-segmented neutrophils, which was not observed in *T. equi* infected horses. Mechanisms underlying the apparent neutropenia are unclear; however, with malaria, the neutropenia may be due to a shift from the circulating

to marginating pool and changes in intravascular granulocyte distribution (Akinosoglou et al., 2012; Dale and Wolff, 1973). Further support for reduced virulence includes lower numbers of observed infected erythrocytes in both spleen-intact and splenectomized horses, minimal changes in hematologic parameters, and survival of a splenectomized horse (Ho-301). The relatively low parasitemia, transitory fever, and survival of Ho-301 without any interventions is in stark contrast to previous reports that have consistently shown that splenectomized horses typically succumb to disease with the development of high parasitemia (> 30%) and significant anemia (Ambawat et al., 1999; De Waal et al., 1988; Guimarães et al., 1997; Kuttler et al., 1986; Wise et al., 2013). Ho-301 is only a single case, and inoculation of additional animals would be needed to confirm that *T. haneyi* is less virulent.

As observed during primary infection with *T. haneyi*, no overt clinical signs were documented in any of the superinfected horses regardless of primary inoculated species. The asymptomatic nature of superinfection resembles the clinical picture observed in *Plasmodium spp*. superinfection cases in endemic regions, and the underlying mechanism limiting the clinical manifestations of disease for *Plasmodium* spp. are unknown but thought to be associated with density-dependent regulation of parasitemia (Bruce and Day, 2002). Additionally with *Plasmodium* spp., density-dependent regulation of parasitemia through regulation of host iron metabolism has been found to inhibit establishment of superinfection (Portugal et al., 2011). Unfortunately, a definitive mechanism has not been defined for *T. equi* at this time, as a heterologous organism would be expected to establish infection uninhibited unless significant cross-reactive immunity existed. Individuals appear to be more susceptible to development of clinical disease if they lack an antibody response to specific surface proteins and become less susceptible with the accumulation of an antibody repertoire (Giha et al., 1999).

Ultimately, the stealth emergence and persistence of *T. haneyi* raises concerns of its prevalence within the U.S. borders, as it is undetectable using currently available diagnostics (Knowles et al., 2018). *T. haneyi's* capacity to co-exist with another species and remain clinically silent could enable a silent reservoir of infectious organisms that could enhance the opportunity for transmission and, additionally, promote competitive evolutionary change. Within-host competition can potentially promote increased virulence as the parasite attempts to maximize its fitness, and therefore potentially elevate the average virulence of a population of parasites above the equilibrium level expected when competition is absent. As a consequence, within-host competition may increase the risk of competitive suppression and favor more deadly organisms (de Roode et al., 2005). More virulent parasites typically have a competitive advantage over more benign strains; however, in some cases, avirulent strains have suppressed virulent strains as is seen with attenuated lines utilized in vaccine development (Bell et al., 2006; Dalgliesh et al., 1981). However, the degree of virulence of *T. haneyi*^{EP} has not been fully defined nor can it be delineated from this study alone.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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