

Article

Development of an indirect ELISA to detect equine antibodies to *Theileria haneyi*

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Abstract:

The apicomplexan parasite *Theileria haneyi* is one of two known causative agents of equine theileriosis. It causes milder clinical disease than its more virulent counterpart, *Theileria equi*, in experimentally infected horses, and can superinfect *T. equi*-positive horses. The current EMA1-based competitive ELISA used in the U.S. to detect equine theileriosis detects *T. equi* but not *T. haneyi*, and the complexity of molecular assays precludes widespread use for epidemiologic studies. In order to facilitate urgently needed studies on the prevalence of *T. haneyi*, the goal of this study was to develop a sensitive and specific serologic assay for the diagnosis of *T. haneyi* based on the equi merozoite antigen 11 (*ThEMA11*). To achieve this objective, *ThEMA11* was recombinantly expressed in eukaryotic cells and its antigenicity assessed using sera from *T. haneyi*-experimentally infected horses. Confirmation of sera reactivity enabled design and optimization of an indirect ELISA. Specificity of the ELISA for *T. haneyi* was assessed using a cohort of sera from horses experimentally infected and confirmed PCR-positive for either *T. equi* or *T. haneyi*. Data from field samples further demonstrate that the *ThEMA11* ELISA is capable of identifying *T. haneyi* antibodies in horses from multiple continents around the world.

Keywords: Equine theileriosis; *Theileria haneyi*; enzyme-linked immunosorbent assay (ELISA), serology

1. Introduction

Theileria haneyi is an apicomplexan hemoparasite and one of two known causative agents of equine theileriosis. *T. haneyi* appears to have a global distribution, with infected equids having been identified in North America, South America, and Africa [1-5]. The organism causes milder clinical disease (variable fever, anemia) than *T. equi* in experimentally infected horses, and is capable of superinfection with *T. equi* [3,6]. Horses remain persistently infected following the acute stage of disease, and these asymptomatic horses are presumed to be reservoirs of infectious organisms for competent tick vectors. Unfortunately, while the antiparasitic drug imidocarb dipropionate (ID) resolves the majority of equine infections with U.S. strains of *T. equi*, *T. haneyi* does not appear to be susceptible to ID, and co-infection of horses with *T. equi* and *T. haneyi* reduces the efficacy of ID against *T. equi* [7].

Initial investigation into the serologic immune response to *T. haneyi* revealed that sera from *T. haneyi*-infected horses react with affinity purified, *T. equi* (Florida isolate) equi merozoite antigens (EMA) 1 and 2 [6]. Interestingly, genomic analysis revealed that the *T. haneyi* genome lacks the *ema1*, 3, and 4 genes, but contains three novel EMA family

members, designated *ema11-13* [3]. Antigenic cross-reactivity is attributed to high amino acid identity within the EMA family, both within the *T. equi* genome and between the *T. equi* and *T. haneyi* genomes [3,8]. The EMA family has garnered significant attention in the veterinary diagnostic community, and regulatory *T. equi* serologic assays approved by The World Organization for Animal Health (OIE) and the United States Department of Agriculture (USDA) are based on the equid immune response to the EMAs. The globally validated EMA1-based *T. equi* competitive ELISA detects a wide range of global isolates [9-11]. However, despite the antigenic cross-reactivity observed via immunoblot, the assay does not detect *T. haneyi* infected horses [3,6]. Currently, PCR-based diagnostic assays are the only available means of definitively confirming *T. haneyi* infection [3,7]. Unfortunately, these molecular diagnostic assays are currently confined to research laboratory use as further optimization is needed to validate the sensitivity of these assays in field-infected animals and to interpret negative results [12].

Due to the aforementioned challenges of molecular assays that currently preclude widespread field use, serology has been the diagnostic assay of choice for detection of infection by the causal agents of equine theileriosis and babesiosis [13]. Therefore, the objective of this study was to develop an indirect ELISA capable of detecting horses infected with *T. haneyi*. To achieve this objective, EMA11, an EMA protein exclusive to *T. haneyi* (*ThEMA11*) was recombinantly expressed and purified. Sera from known *T. haneyi*-infected and uninfected horses were utilized for assessment of specific antibody reactivity with recombinant *ThEMA11*. Confirmation of sera reactivity enabled assay optimization, and indirect ELISA performance was screened against a cohort of horses experimentally infected with either *T. equi* or *T. haneyi* to assess specificity. Data from field samples further demonstrate the *ThEMA11* indirect ELISA can identify *T. haneyi* antibodies in horses from multiple continents.

2. Results

2.1. Cloning and expression of *ThEMA11*

The nucleotide sequence of *ThEMA11* was codon-optimized for mammalian cell expression, and successfully cloned into pcDNA3.1 (Figure 1a). The recombinant plasmid (pcDNA3.1-*ThEMA11*) was sequenced to confirm the presence of *ThEMA11* in-frame with the CMV promoter (data not shown). Subsequently, HEK 293t cells were transiently transfected with pcDNA3.1-*ThEMA11*, and expression of recombinant *ThEMA11* was initially confirmed by immunoblot using the anti-6xHis monoclonal antibody (Figure 1b). Results demonstrated the expression of an approximately 33KDa protein, which is the expected molecular weight of *ThEMA11* fused with the 6xHis tag. No protein with similar molecular weight was detected in control cells transfected with pcDNA3.1-GFP (Figure 1b). Additional protein bands with molecular weight ranging from 38 to >80KDa are also shown in Figure 1b; however, as they are present in both cells transfected with pcDNA3.1-*ThEMA11* or pcDNA3.1-GFP, these are likely non-specific reactions. Once *ThEMA11* was successfully expressed, we next sought to evaluate the antigenicity of this recombinant *T. haneyi* protein using serologic assays.

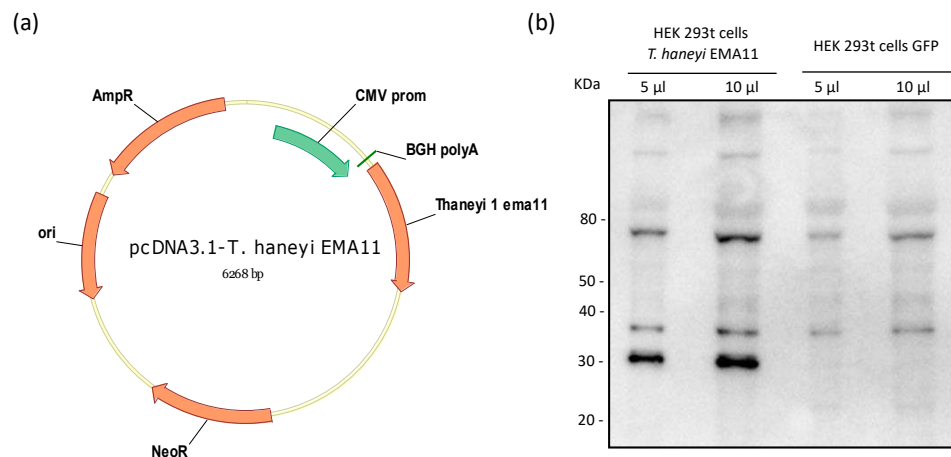


Figure 1. Cloning and expression of *T. haneyi* EMA11 (*ThEMA11*). (a) Codon-optimized sequence of *ThEMA11* was cloned into pcDNA3.1 in frame with the CMV promoter and fused with the 6His tag originating the plasmid pcDNA3.1-*ThEMA11*. (b) Expression of recombinant *ThEMA11* in HEK 293t cells detected by anti-6xHis monoclonal antibody.

2.2. Recombinant *ThEMA11* is recognized by serum from *T. haneyi*-infected horses, but not serum from *T. equi*-infected or uninfected horses

After demonstrating expression of *ThEMA11* by transiently transfected HEK 293t cells, we next examined whether sera from *T. haneyi*-infected horses recognize the recombinant protein expressed by eukaryotic cells. A crude lysate of the pcDNA3.1-*ThEMA11*-transfected HEK 293t cells was used as antigen for immunoblot analysis. Prior to use in immunoblots, horse sera were adsorbed with HEK 293t-cell lysate to decrease non-specific binding, as described in the Materials and Methods section. Results demonstrated that sera from a horse experimentally infected with *T. haneyi* recognized recombinant *ThEMA11* (rec *ThEMA11*) (Figure 2a). Sera from uninfected horses did not detect proteins with the expected molecular weight of *ThEMA11* (representative uninfected horse serum is shown in Figure 2b). Importantly, sera from *T. equi*-infected horses did not show cross reactivity with *ThEMA11* (Figure 2c), despite the high level of amino acid identity between the *T. haneyi* protein and members of the EMA family in *T. equi* (Figure S2). The specific recognition of recombinant *ThEMA11* by *T. haneyi*-infected horse sera, and the absence of recognition of *ThEMA11* by either uninfected or *T. equi*-infected horse sera prompted us to move forward and use recombinant *ThEMA11* to develop an indirect ELISA to detect horse antibodies to *T. haneyi*.

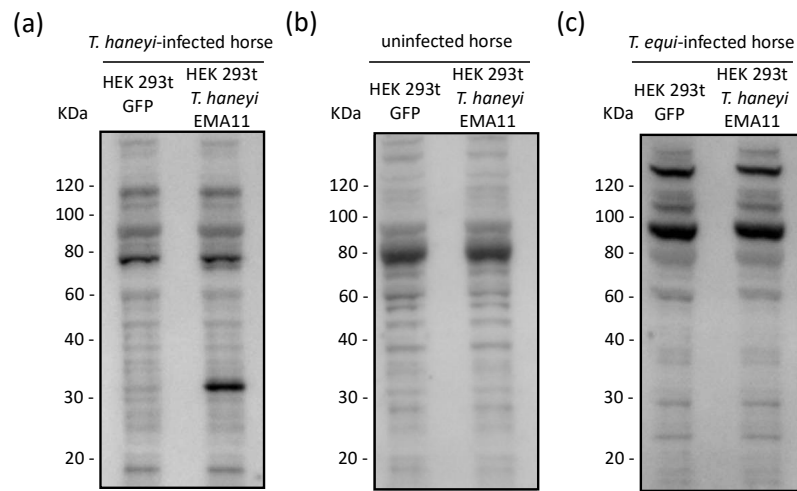


Figure 2. Immunoblot analyses to evaluate the antigenicity of recombinant *ThEMA11* using sera from horses experimentally infected with *T. haneyii* (a), sera from uninfected horses (b), and sera from *T. equi* infected horses (c). HEK 293t cells expressing GFP were used as a negative control.

2.3 Optimization of *ThEMA11* as an antigen for use in the indirect ELISA format

As a first step for ELISA optimization, we scaled up the transient transfection of HEK 293t cells with pcDNA3.1-*ThEMA11* and purified the recombinant *ThEMA11* using cobalt resin. The level of purity of recombinant *ThEMA11* was assessed following each step of the purification process by immunoblot using a monoclonal antibody to the anti-6xHis tag (data not shown). A concentration of approximately 1 µg/µl of rec *ThEMA11* was obtained after purification. Next, the antigenicity of purified *ThEMA11* was evaluated using sera from *T. haneyii*-infected horses (Figure 3). The results of this analysis demonstrate that purified, recombinant *ThEMA11* retained its antigenicity and was specifically recognized by sera from *T. haneyii*-infected horses. Immunoblots using sera from two representative, *T. haneyii* experimentally infected horses, Ho-344 and Ho-777, are in shown in Figure 3. Sera from uninfected horses failed to react with purified, recombinant *ThEMA11*. Immunoblots using sera from two representative uninfected animals, Ho-305 and Ho-404, are presented in Figure 3. Similarly, no reactivity was observed when anti-horse IgG HRP secondary antibodies were used alone (Figure 3).

Subsequently, the optimal amount of recombinant *ThEMA11* was determined in an ELISA format in relation to horse serum dilutions (Figure 4). For this analysis, we used serum from one representative *T. haneyii*-infected horse and serum from one representative uninfected horse. Four serial dilutions of the sera (1:8, 1:16, 1:32, and 1:64), and four different *ThEMA11* recombinant protein concentrations (5 µl/well, 4 µl/well, 2 µl/well, and 1 µl/well) were tested (Figure 4a-d). Data showed that the best discrimination between positive and negative sera was obtained using 4 µl/well of antigen with a serum dilution of 1:8 (Figure 4b). Therefore, these parameters were used for the *ThEMA11*-based ELISA. OD450nm values below 0.2 were obtained when rabbit anti-horse IgG HRP was used alone (data not shown). In addition, we used the *Rhipicephalus microplus* tick antigen Bm86 fused with the 6-His tag (Bm86-6-His) and purified by cobalt column, a similar approach used to purify *ThEMA11*, to evaluate potential reactivity of horse antibodies to the 6-His tag. No significant reactivity was detected in representative *T. haneyii*-infected and uninfected horse sera against Bm86-6-His, indicating that that 6-His tag is not implicated in the OD signal observed in the *ThEMA11* ELISA (data not shown).

A total of 18 serum samples from *T. haneyii* experimentally infected horses and 19 serum samples from uninfected horses were then used to determine the *ThEMA11*-based ELISA cutoff (Figure 5). An optimal cutoff of 0.8

OD_{450nm} was determined, representing the average of negative samples plus three standard deviations. In addition, sera from nine experimentally *T. equi*-infected, known *T. haneyi*-negative horses were also analyzed. As demonstrated, sera from all *T. equi*-infected horses present OD values below the cutoff threshold and therefore, were correctly shown as negative for exposure to *T. haneyi* (Figure 5). Assessment of 35 field-collected horse serum samples (11 known *T. haneyi*-infected and 24 known uninfected horses) demonstrated that the recombinant *Th*EMA11-based ELISA correlates well with immunoblot results and showed relative sensitivity and specificity of the new ELISA of 90.90 and 95.83%, respectively.

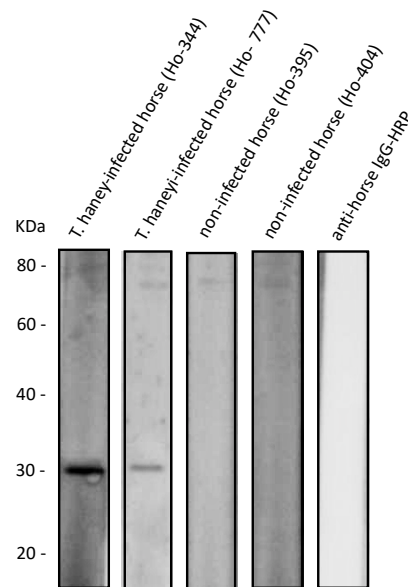


Figure 3. Antigenicity of purified recombinant *Th*EMA11 using sera from *T. haneyi*-infected horses (Ho-344 and Ho-777). Sera from uninfected horses (Ho-395 and Ho-404) were used as negative controls. Rabbit anti-horse IgG-HRP alone was also used as a negative control.

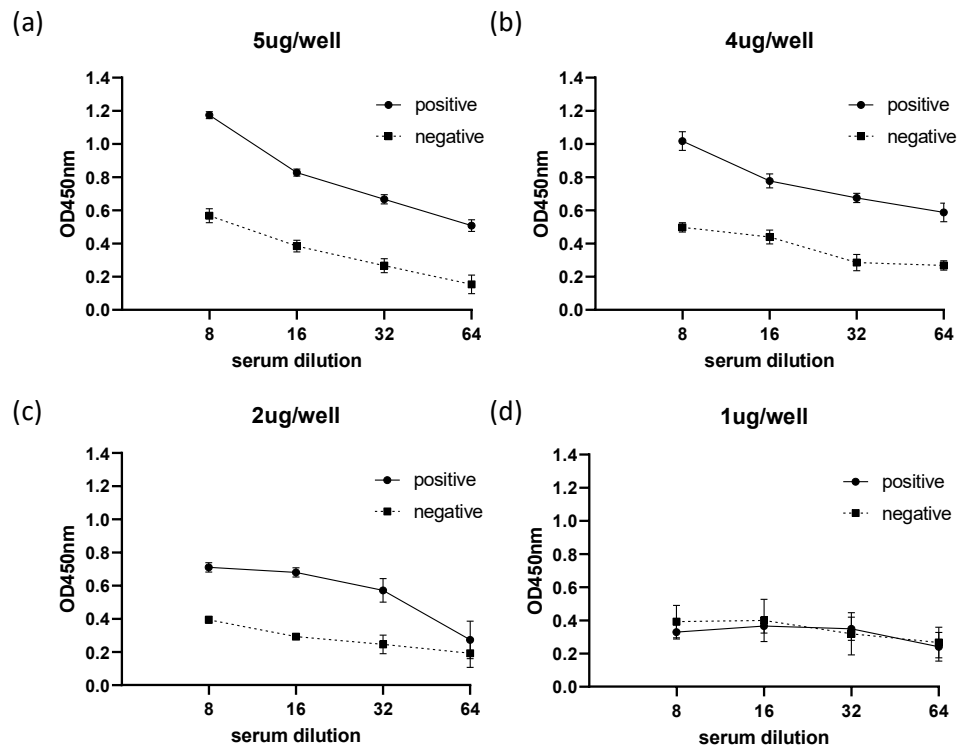


Figure 4. Titration of the optimal amount of recombinant *ThEMA11* in an ELISA format in relation to horse serum dilutions. Results show titration of serum from one representative *T. haneyi*-infected horse and serum from one representative uninfected horse. Four serial dilutions of the sera (1:8, 1:16, 1:32, and 1:64), and four different *ThEMA11* recombinant protein concentrations (5 µl/well, 4 µl/well, 2 µl/well, and 1 µl/well) were tested (a-d).

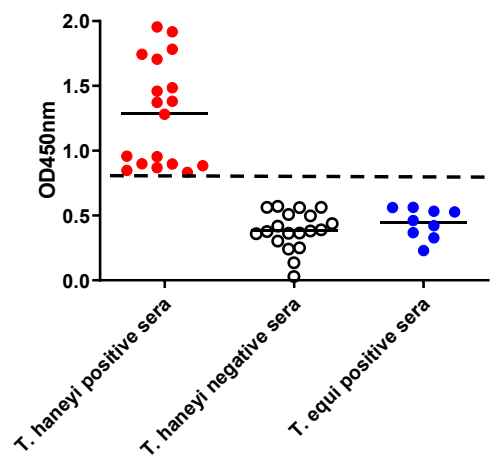


Figure 5. Results of the *ThEMA11*-based ELISA using serum samples (n=18) from *T. haneyi* experimentally infected horses (n=14), from *T. equi* experimentally infected horses (n=9), and from uninfected horses (n=19). Dashed line indicates the cutoff of 0.8 OD_{450nm} representing the average of negative samples (uninfected horse sera) plus three standard deviations.

2.4. Use of the *Th*EMA11-based ELISA to detect antibodies to *T. haneyi* in horse sera from different geographic regions

After determining the positive threshold, sensitivity, and specificity of the *Th*EMA11-based ELISA, we used the assay to investigate the presence of antibodies against *T. haneyi* in serum samples from horses residing in or with a travel history to distinct geographical regions (Figure 6). A total of 176 horse sera were tested (145 from U.S., 6 from Germany, 13 from Mexico, 2 from France, 3 from Ireland, 1 from Puerto Rico, 3 from the Netherlands, and three sera from unknown locations). Of the tested samples, 50.56% (n=90) were positive for *T. haneyi* using the *Th*EMA11 ELISA, and 48.86% (n=86) were considered negative (Figure 6). These equine serum samples were also assessed for *T. equi* and *B. caballi* antibodies (Table 1 and 2). Results demonstrated that 58.62% (51 serum samples) were positive for both *T. haneyi* EMA11 and *T. equi* EMA1. In contrast, 62.96% (51 serum samples) were negative for *Th*EMA11 but positive for the presence of antibodies to *T. equi* EMA1. A total of 27.92% (7 serum samples) were positive both for *Th*EMA11 and *B. caballi* RAP-1 antibodies. On the contrary, 29.41% (5 serum samples) were positive for the presence of *B. caballi* RAP-1 antibodies, but negative for *Th*EMA11. Considering the detection of *Th*EMA11 antibodies in samples that were negative for *T. equi* EMA1 or *B. caballi* RAP-1, together the results also reinforce the specificity of the *Th*EMA11-based ELISA described in this study. Collectively, the results demonstrate the use of the *Th*EMA11 ELISA to investigate the presence of antibodies to *T. haneyi* in horse sera from distinct geographical regions around the world.

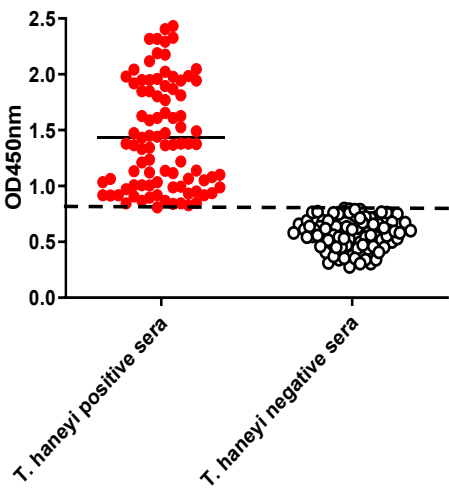


Figure 6. Use of the *Th*EMA11-based ELISA to investigate the presence of antibodies against *T. haneyi* in serum samples (n=176) from horses residing in or with a travel history to distinct geographical regions endemic for equine theileriosis. Dashed line indicates the cutoff of 0.8 OD450nm previously determined representing the average of negative samples (uninfected horse sera) plus three standard deviations.

Table 1. Presence of *T. haneyi* EMA11 and *T. equi* EMA1 antibodies in horse sera from distinct geographic regions.

| <i>T. haneyi</i> positive samples (n=87) | | <i>T. haneyi</i> negative samples (n=81) | |
|--|-------------------------|--|-------------------------|
| <i>T. equi</i> positive | <i>T. equi</i> negative | <i>T. equi</i> positive | <i>T. equi</i> negative |
| 58.62% (n=51) | 41.37% (n=36) | 62.96% (n=51) | 37.07% (n=30) |

Table 2. Presence of *T. haneyi* EMA11 and *B. caballi* RAP-1 antibodies in horse sera from distinct geographic regions.

| <i>T. haneyi</i> positive samples (n=26) | | <i>T. haneyi</i> negative samples (n=17) | |
|--|----------------------------|--|----------------------------|
| <i>B. caballi</i> positive | <i>B. caballi</i> negative | <i>B. caballi</i> positive | <i>B. caballi</i> negative |
| 26.92% (n=7) | 73.07% (n=19) | 29.41% (n=5) | 70.58% (n=12) |

3. Discussion

Currently, the only available diagnostic assay for *T. haneyi* is nested PCR, which hinders widespread assessment of prevalence of this recently discovered organism. Despite the reported high sensitivity of PCR, especially when utilizing nested PCR, multiple factors can lead to inhibition of reactions, leading to false negative results [14]. Another downside of direct molecular diagnostic assays, such as PCR, is the potential for false-negative results due to low parasitemia in peripheral blood, especially in the chronic phase of *B. caballi* infections. In addition, molecular assays are relatively more expensive. Therefore, serology remains the more widely accepted diagnostic, especially for persistent infections such as those caused by *T. equi* and *T. haneyi*.

Since *T. equi* and *T. haneyi* are closely related, careful analysis was required to identify a candidate antigen for use in a *T. haneyi*-specific diagnostic assay. In a previous study, antigenic cross-reactivity was observed to a subset of *T. equi* proteins in immunoblots, but the *T. equi* EMA1 based regulatory diagnostic competitive ELISA failed to detect *T. haneyi* infected horses. For development of a *T. haneyi*-specific serologic assay, the *T. haneyi* EMA11 protein was selected because it is exclusive to *T. haneyi*. The antigen was then carefully evaluated to ensure cross-reactivity did not occur between *T. equi* and *T. haneyi* infected horses as there is a high level of amino acid identity between the *T. equi* and *T. haneyi* EMA family members. Additionally, a mammalian cell expression system was utilized to ensure proper protein folding, and to reduce bacterial protein contamination and resultant high background reactivity. Following expression and purification, the antigenicity of recombinant *Th*EMA11 was verified via screening with sera from known infected and non-infected horses. The infection status of these horses was correlated at multiple timepoints with nPCR and/or serial blood smears, and immunoblot using serum from horses in both the acute and chronic stages of infection.

Since serum from horses experimentally infected with only one geographic isolate of *T. haneyi* was used for the initial assay development and optimization steps, we chose to screen a cohort of geographically diverse equine serum samples to gain a broader perspective of the potential use of this assay on a global scale. Performance of the assay using this diverse sample set was strong, and high agreement was noted with the *T. haneyi* immunoblot. Using this information, the serologic assay was determined to have a sensitivity and specificity of 90.9% and 95.83%, respectively. It was beyond the scope of this study to investigate the level of conservation of *Th*EMA11 in parasites around the world. However, the fact that the *Th*EMA11-based ELISA was able to detect antibodies in geographically diverse equine serum samples suggest that EMA11 is highly immunogenic and conserved among worldwide isolates of *T. haneyi*.

A reliable serologic diagnostic assay for *T. haneyi* will become crucial for international movement of horses as recently published data suggests that co-infection with *T. haneyi* reduces the efficacy of ID against *T. equi* [7]. Furthermore, ID lacks efficacy in horses infected with *T. haneyi* alone [7]. This data is concerning, as potential new anti-*Theileria* chemotherapeutic compounds are currently only in early testing phase, leaving a paucity of potential therapeutic options for infected horses [15-17]. Additionally, although experimental infection with *T. haneyi* appears to be mild, the clinical signs and severity associated with natural infection remains unknown, and could be more significant, as has been documented with *T. equi*.

The assay developed in this study will enable subsequent, global evaluation of the prevalence of *T. haneyi*. Following more extensive validation, use of this assay in screening horses prior to inter-country movement may help with the continued control on equine theileriosis on a global scale.

4. Materials and Methods

4.1. *T. haneyi* EMA11 cloning, expression, and purification

T. haneyi parasites and the *ThEMA11* sequence used in this study were previously described in [3,8]. The full-length sequence of *ThEMA11* was codon-optimized for mammalian cells, and a 6His tag added to its C-terminal end (GeneArt, Thermo Fisher Scientific) (Figure S1). The synthetic gene was then cloned into pcDNA3.1 and the plasmid, termed pcDNA3.1-*ThEMA11*, was then used to express the target protein in human embryonic kidney (HEK) 293t cells (ATCC®).

For the expression of recombinant *ThEMA11*, HEK 293t cells were transiently transfected with pcDNA3.1-*ThEMA11* using polyethylenimine (PEI) transfection reagent per standard protocols. Briefly, HEK 293t cells were seeded overnight in six-well plates (70 to 80% confluence) and transfected with a target plasmid using PEI (1 µg plasmid DNA: 4 µl PEI ratio), as previously described [18]. Four hours after transfection, the transfection mix was replaced with complete Dulbecco's modified essential medium (cDMEM) (10% fetal bovine serum, 24 mM of HEPES, 2 mM of L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin). 48 hours after transfection, cells were collected in 1x Cell Culture Lysis Reagent (Promega) containing the Halt Protease Inhibitor Single Use Cocktail, EDTA-Free (Thermo Fisher Scientific). Lysate of transfected HEK 293t cells were stored at -80°C until use for immunoblot analysis and protein purification.

Recombinant *ThEMA11* was purified using the HisPur™ Cobalt Purification Kit following the manufacturer's protocol (Thermo Fisher Scientific). Briefly, transient transfection of HEK 293t was scaled up to be performed in 150 cm² cell culture flasks. Forty-eight hours post-transfection, the culture supernatant was removed, and cells re-suspended in 1x PBS containing Halt Protease Inhibitor Single Use Cocktail, EDTA-Free. Cells were then lysed in three freeze-thaw cycles and stored at -80°C until purification. For protein purification, cobalt columns were equilibrated to room temperature and washed in Equilibration/Wash Buffer provided by the HisPur™ purification kit. The lysate of pcDNA3.1-*ThEMA11*-transfected HEK 293t cells was loaded into the columns and incubated for 30 minutes at 4°C on a rocking platform. Columns were then centrifuged at 700 *xg* for 2 minutes, and the resin washed twice in Equilibration/Wash Buffer. Target protein was eluted by adding one resin-bed volume of Elution Buffer provided by the HisPur™ purification kit followed by centrifugation at 700 *xg* for 2 minutes. Elution steps were repeated twice, and fractions were stored at -80°C until use.

4.2. Horse serum samples

Serum samples from *T. haneyi* experimentally infected horses (n=13) were collected at multiple time points following infection, and used as positive controls. Serum samples (n=19) from healthy horses located in WA or ID, USA (where *T. equi* and *T. haneyi* are not naturally present in equine populations), which were confirmed negative for *T. haneyi* via nPCR and blood smear cytology were used as negative controls in this study. The known *T. haneyi*-positive and negative sera were used to determine the *ThEMA11*-based ELISA cutoff. In addition, serum samples from confirmed *T. equi*-infected horses (n=8) were also used to assess the specificity of the *T. haneyi* EMA11-based ELISA. All serum samples were obtained from horses housed at the USDA-ADRU, University of Idaho, or Washington State University animal facilities. All animal experiments were approved by the Washington State University and University of

Idaho Institutional Animal Care and Use Committees, ASAF numbers 4973 and 6241 (Washington State University) and 2016-18 and 2016-28 (University of Idaho).

Field horse serum samples (n=176) were obtained from different regions around the world, including the U.S., Mexico, and Germany, among other countries. Serum samples were obtained through collaboration with OIE Reference Laboratory for equine piroplasmiasis located at Washington State University. Reactivity of these samples for *T. equi* and/or *B. caballi*, depending on the individual case circumstances, was determined by the Reference Laboratory via commercially available cELISAs and immunoblots as described below. The date of collection and sample source location are presented in Table S1.

4.3. Competitive ELISA (cELISA) for *T. equi* or *B. caballi*

Horse sera tested in this study for the presence of anti-*T. haneyi* antibodies were evaluated by cELISA to detect antibodies to *T. equi* and/or *B. caballi* as previously described in Knowles et al 1992 and Kappmeyer et al 1999, respectively [19,20].

4.4. Immunoblot analysis

Immunoblots were performed using lysates of HEK 293t transfected with pcDNA3.1-*ThEMA11* or with purified recombinant *ThEMA11* as antigen and sera from known *T. haneyi*-infected horses or monoclonal antibody (mAb) recognizing the 6His tag (clone AD1.1.10; Bio-Rad). In addition, sera from healthy, uninfected horses and confirmed *T. equi*-infected horses were used as controls in the immunoblots. Briefly, 5 or 10 µl of cell lysate or 5 µl of recombinant purified *ThEMA11* was loaded into each lane and separated in NuPAGE™ 4-12% Bis-Tris gel (Invitrogen). The gel was then electrotransferred to a nitrocellulose membrane and blocked with PBS 0.02% Tween 20 (PBS-T) 10% non-fat milk. After blocking, the membrane was incubated for 1 hour at room temperature with either horse serum (1:50 or 1:100) or the anti-6His tag mAb (1:500). For immunoblots using lysate of HEK 293t cells transfected with pcDNA3.1-*ThEMA11*, horse sera were adsorbed as follows. Serum samples were incubated for 48 hours at 4°C with lysate of wildtype HEK 293 cells (approximately 10⁶ cells). After that, the mix was centrifuged (1,000 xg, 5 minutes) and supernatant used for the immunoblots. After washing in PBS-T, the membrane was incubated for 1 hour at room temperature with secondary anti-mouse IgG HRP (SeraCare; 1:2,500) or anti-horse IgG HRP (SeraCare; 1:2,500). The immune complexes were revealed using an enhanced chemiluminescence method (ECL™; Amersham).

4.5. Indirect ELISA

Immunolon™ 2 HB 96-well microtiter ELISA plates (Thermo Scientific) were coated overnight at 4°C with 50 µl of recombinant purified *ThEMA11* (4µg/well) in 1x Coating Buffer (BioLegend). After that, excess antigen was removed, and the plates were blocked with 200 µl/well of Blocker™ Casein in PBS (Thermo Fisher Scientific) at room temperature (RT) for 1 hour. Following the blocking step, serial dilutions (1:2 to 1:256) of *T. haneyi*-positive or negative horse sera were added to the plates and incubated at RT for 1 hour. After five washes in 0.05% (v/v) Tween-20 in PBS (PBS-T), rabbit anti-horse IgG-HRP (1:10,000) (Millipore-Sigma) was added to each well, and the plates were incubated at RT for 1 hour. Plates were then washed five times in PBS-T and developed with 100 µl of 1-Step™ Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific). The enzymatic reaction was stopped via addition of 100 µl of TMB Stop Solution (0.2 M H₂SO₄) (SeraCare) to each well, and plates read at 450 nm using an ELISA plate reader (MultiSkan MCC, Thermo Fisher Scientific). Immunoblot was used as gold standard to evaluate sensitivity and specificity of the *ThEMA11*-based ELISA. Sensitivity and specificity were calculated using the following formula: sensitivity=[number of true positive

samples-number of false negative samples)x100]/ number of true positive samples; and specificity=[number of true negative samples-number of false positive samples)x100]/number of true negative samples.

Supplementary Materials: Figure S1: Codon-optimized *Theileria haneyi* EMA11 for mammalian expression. Figure S2: Amino acid alignment of *Theileria haneyi* EMA11 and *T. equi* EMA1. Table S1: List of field horse samples.

Author Contributions: Conceptualization, L.F., R.B., M.U., and D.K.; methodology, L.F., R.B., L.K., M.U., and D.K.; formal analysis, L.F., R.B., L.K., D.K.; investigation, R.B., K.S., L.K., and K.D.; resources, L.F., M.U., and D.K.; data curation, R.B.; writing—original draft preparation, R.B., K.S., and L.F.; writing—review and editing, L.K., M.U., D.K. and K.D.; visualization, R.B.; supervision, L.F., M.U., and D.K.; project administration, L.F., D.K. and M.U.; funding acquisition, D.K., L.F., and M.U. All authors have read and agreed to the published version of the manuscript.

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References

1. Bishop, R.P.; Kappmeyer, L.S.; Onzere, C.K.; Odongo, D.O.; Githaka, N.; Sears, K.P.; Knowles, D.P.; Fry, L.M. Equid infective *Theileria* cluster in distinct 18S rRNA gene clades comprising multiple taxa with unusually broad mammalian host ranges. *Parasit Vectors* **2020**, *13*, 261,doi: 10.1186/s13071-020-04131-0.
2. Bhoora, R.V.; Collins, N.E.; Schnittger, L.; Troskie, C.; Marumo, R.; Labuschagne, K.; Smith, R.M.; Dalton, D.L.; Mbizeni, S. Molecular genotyping and epidemiology of equine piroplasmids in South Africa. *Ticks Tick Borne Dis* **2019**, 101358,doi: 10.1016/j.ttbdis.2019.101358.
3. Knowles, D.P.; Kappmeyer, L.S.; Haney, D.; Herndon, D.R.; Fry, L.M.; Munro, J.B.; Sears, K.; Ueti, M.W.; Wise, L.N.; Silva, M., *et al.* Discovery of a novel species, *Theileria haneyi* n. sp., infective to equids, highlights exceptional genomic diversity within the genus *Theileria*: implications for apicomplexan parasite surveillance. *Int J Parasitol* **2018**, doi: 10.1016/j.ijpara.2018.03.010.
4. Vieira, M.I.B.; Costa, M.M.; de Oliveira, M.T.; Goncalves, L.R.; Andre, M.R.; Machado, R.Z. Serological detection and molecular characterization of piroplasmids in equids in Brazil. *Acta Trop* **2018**, *179*, 81-87,doi: 10.1016/j.actatropica.2017.12.028.
5. Mshelia, P.W.; Kappmeyer, L.; Johnson, W.C.; Kudi, C.A.; Oluyinka, O.O.; Balogun, E.O.; Richard, E.E.; Onoja, E.; Sears, K.P.; Ueti, M.W. Molecular detection of *Theileria* species and *Babesia caballi* from horses in Nigeria. *Parasitology research* **2020**, *119*, 2955-2963,doi: 10.1007/s00436-020-06797-y.
6. Sears, K.P.; Kappmeyer, L.S.; Wise, L.N.; Silva, M.; Ueti, M.W.; White, S.; Reif, K.E.; Knowles, D.P. Infection dynamics of *Theileria equi* and *Theileria haneyi*, a newly discovered apicomplexan of the horse. *Vet Parasitol* **2019**, *271*, 68-75,doi: 10.1016/j.vetpar.2019.06.009.

7. Sears, K.; Knowles, D.; Dinkel, K.; Mshelia, P.W.; Onzere, C.; Silva, M.; Fry, L. Imidocarb Dipropionate Lacks Efficacy against *Theileria haneyi* and Fails to Consistently Clear *Theileria equi* in Horses Co-Infected with *T. haneyi*. *Pathogens* **2020**, *9*, doi: 10.3390/pathogens9121035.
8. Wise, L.N.; Kappmeyer, L.S.; Knowles, D.P.; White, S.N. Evolution and diversity of the EMA families of the divergent equid parasites, *Theileria equi* and *T. haneyi*. *Infect Genet Evol* **2019**, *68*, 153-160,doi: 10.1016/j.meegid.2018.12.020.
9. Knowles, D.P., Jr.; Perryman, L.E.; Goff, W.L.; Miller, C.D.; Harrington, R.D.; Gorham, J.R. A monoclonal antibody defines a geographically conserved surface protein epitope of *Babesia equi* merozoites. *Infect Immun* **1991**, *59*, 2412-2417,
10. Cunha, C.W.; Kappmeyer, L.S.; McGuire, T.C.; Dellagostin, O.A.; Knowles, D.P. Conformational Dependence and Conservation of an Immunodominant Epitope within the *Babesia equi* Erythrocyte-Stage Surface Protein Equi Merozoite Antigen 1. *Clin Vaccine Immunol* **2002**, *9*, 1301-1306,doi: 10.1128/cdli.9.6.1301-1306.2002.
11. Ueti, M.W.; Palmer, G.H.; Kappmeyer, L.S.; Scoles, G.A.; Knowles, D.P. Expression of Equi Merozoite Antigen 2 during Development of *Babesia equi* in the Midgut and Salivary Gland of the Vector Tick *Boophilus microplus*. *Journal of Clinical Microbiology* **2003**, *41*, 5803-5809,doi: 10.1128/jcm.41.12.5803-5809.2003.
12. Wise, L.N.; Kappmeyer, L.S.; Silva, M.G.; White, S.N.; Grause, J.F.; Knowles, D.P. Verification of post-chemotherapeutic clearance of *Theileria equi* through concordance of nested PCR and immunoblot. *Ticks Tick Borne Dis* **2018**, *9*, 135-140,doi: 10.1016/j.ttbdis.2017.08.007.
13. Wise, L.N.; Kappmeyer, L.S.; Mealey, R.H.; Knowles, D.P. Review of equine piroplasmiasis. *J Vet Intern Med* **2013**, *27*, 1334-1346,doi: 10.1111/jvim.12168.
14. Schrader, C.; Schielke, A.; Ellerbroek, L.; Johne, R. PCR inhibitors - occurrence, properties and removal. *J Appl Microbiol* **2012**, *113*, 1014-1026,doi: 10.1111/j.1365-2672.2012.05384.x.
15. Silva, M.G.; Villarino, N.F.; Knowles, D.P.; Suarez, C.E. Assessment of Draxxin® (tulathromycin) as an inhibitor of in vitro growth of *Babesia bovis*, *Babesia bigemina* and *Theileria equi*. *Int J Parasitol Drugs Drug Resist* **2018**, *8*, 265-270,doi: 10.1016/j.ijpddr.2018.04.004.
16. Bowden, G.D.; Reis, P.M.; Rogers, M.B.; Bone Relat, R.M.; Brayton, K.A.; Wilson, S.K.; Di Genova, B.M.; Knoll, L.J.; Nepveux, V.F.; Tai, A.K., et al. A conserved coccidian gene is involved in *Toxoplasma* sensitivity to the anti-apicomplexan compound, tartrolon E. *Int J Parasitol Drugs Drug Resist* **2020**, *14*, 1-7,doi: 10.1016/j.ijpddr.2020.07.003.
17. O'Connor, R.M.; Nepveux, V.F.; Abenoja, J.; Bowden, G.; Reis, P.; Beaushaw, J.; Bone Relat, R.M.; Driskell, I.; Gimenez, F.; Riggs, M.W., et al. A symbiotic bacterium of shipworms produces a compound with broad spectrum anti-apicomplexan activity. *PLoS Pathog* **2020**, *16*, e1008600,doi: 10.1371/journal.ppat.1008600.
18. Bastos, R.G.; Sears, K.; Dinkel, K.D.; Knowles, D.P.; Fry, L.M. Changes in the Molecular and Functional Phenotype of Bovine Monocytes during *Theileria parva* Infection. *Infect Immun* **2019**, *87*, doi: 10.1128/iai.00703-19.
19. Knowles, D.P.; Kappmeyer, L.S.; Stiller, D.; Hennager, S.G.; Perryman, L.E. Antibody to a Recombinant Merozoite Protein Epitope Identifies Horses Infected with *Babesia equi*. *J Clin Microbiol* **1992**, *30*, 3122-3126,
20. Kappmeyer, L.S.; Perryman, L.E.; Hines, S.A.; Baszler, T.V.; Katz, J.B.; Hennager, S.G.; Knowles, D.P. Detection of equine antibodies to *Babesia caballi* by recombinant *B. caballi* rhoptry-associated protein 1 in a competitive-inhibition enzyme-linked immunosorbent assay. *J Clin Microbiol* **1999**, *37*, 2285-2290,doi: 10.1128/jcm.37.7.2285-2290.1999.