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Repertoire of *Theileria equi* immunodominant antigens bound by equine antibody

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ABSTRACT

Theileriosis in horses and cattle is caused by tick-borne Apicomplexa parasites and results in death or lifelong infection in their respective hosts. Transmission risk associated with persistent infection severely limits movement of horses and cattle resulting in economic losses. The recent reemergence of *Theileria equi* infection in U.S. horses demonstrates the continual threat Apicomplexa parasites represent to global animal health. A paucity of data concerning equine immune responses to *T. equi*, including antigens recognized by antibodies in clinically asymptomatic, persistently infected horses, precludes vaccine development. Therefore, this investigation was initiated to characterize antigens recognized by the equine antibody response to *T. equi*. This goal was accomplished by defining *T. equi* merozoite antigens that are recognized by antibodies in horses infected with distinct *T. equi* isolates. Previously it was shown that equine post-infection serum consistently recognized at least five *T. equi* merozoite antigens, but their precise identity remained unknown. To determine specificity of antibody target identification, *T. equi* merozoite antigens were first isolated using equine post-infection serum in affinity chromatography. Proteins recognized by the equine antibodies were then isolated from two-dimensional electrophoresis gels, and analyzed by liquid chromatography tandem mass spectrometry (LC–MS/MS) using the recently available *T. equi* genome database. Five *T. equi* antigens were identified and include Equi Merozoite Antigen-2 (EMA-2), EMA-3 and EMA-6, a previously uncharacterized protein annotated as "signal peptide containing protein", and 40S ribosomal protein S12.

1. Introduction

Theileria equi is an Apicomplexa tick-borne parasite responsible for causing equine theileriosis. Infection occurs in tropical, subtropical and temperate regions of the world, including Asia, Europe, Africa and America, and is associated with geographic distribution and seasonal activity of the ixodid ticks *Rhipicephalus* sp., *Dermacentor* sp., and *Hyalomma* sp. [1]. More recently, *Amblyoma cajennense* was also identified as a competent vector for *T. equi* in the USA [2]. Pathogenesis is characterized by initial infection of peripheral blood mononuclear cells by *T. equi* sporozoites followed by infection and lysis of erythrocytes by merozoites [3]. *T. equi* causes acute, sub-acute, and persistent infection disease. Infection of horses with *T. equi* results in fever and anemia; however, lymphocyte proliferation and/or transformation, as occurs in infection of cattle with *T. parva*, are not detectable in *T. equi* infection [4]. Acute infection of immunologically naïve horses with *T. equi* may result in death [1], and available data indicate that horses which survive acute infection become persistently infected and thus lifelong reservoirs for transmission [5,6]. Persistent infection is characterized by fluctuation in the levels of parasitized erythrocytes between $10^{3.3}$ to $10^{6.0}$ parasites/ml of blood [7]. Persistent infection with *T. equi* is characterized by nonspecific signs such as weight loss and poor body condition.

The recent detection of *T. equi* infection in 292 out of a total of 360 horses in Southern Texas, and the involvement of the newly discovered *A. cajennense* vector, provides a clear example of the insidious nature of the reemergence of a vector borne infection and the role of parasite persistence in transmission [8]. In the absence of a vaccine, the control of equine theileriosis is performed essentially from two ways: restricting importation of infected horses (mainly in non-endemic regions) and, by the detection of infected horses followed either by euthanasia or treatment with chemotherapeutics aimed at elimination of infection and transmission risks, or by maintenance of premunition, the immunity associated by persistent infection (mainly in endemic regions). Treatment to eliminate infection and transmission risk of horses persistently infected with

Abbreviations: EMA, equi merozoite antigen; 1-2D, one or two dimensional electrophoresis; LC–MS/MS, liquid chromatography tandem mass spectrometry; cELISA, competitive ELISA; nPCR, nested PCR.

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T. equi with either buparvaquone [9] or high-dose of imidocarb dipropionate [10] has not provided consistent results. However, a recent study has shown that imidocarb dipropionate eliminates persistent *T. equi* infection in infected horses from Southern Texas [11]. The study reported here was initiated to identify targets of the equine antibody response, with the intent of improving available methodologies for detection and control of equine theileriosis as well as the potential discovery of new vaccine candidates for *T. equi*.

The fluctuation in parasite levels characterized in *T. equi* infections [7] is suggestive of antigenic variation within the merozoite population. This pattern of control and emergence of parasitemia is well known and characteristic in parasites that undergo antigenic variation with one of the most well studied examples being *Trypanosoma brucei* [12]. Two extensively studied *T. equi* merozoite surface proteins, belonging to the *T. equi* equi merozoite antigens (EMA) family (EMA-1 and EMA-2) are known to elicit antibody during *T. equi* infection in horses [13]. Both, EMA-1 and EMA-2 proteins have been identified as immunodominant antigens [14–16] that share 52% amino acid identity. They also share a glycosyl-phosphatidylinositol (GPI) anchor motif, indicating that these proteins might be expressed on the outer membrane of merozoite surface with a GPI anchor [16,17]. In addition, other *T. equi* antigens were detected previously, but their precise identity unknown [18–25]. Studies demonstrated several *T. equi* antigens recognized by *T. equi* infected horses, with molecular masses ranging from 18 to 96 kDa [18], 19 to 33 kDa [19] and, 30 to 34 kDa [23].

In this study, affinity chromatography with equine post-infection antibody and liquid chromatography tandem mass spectrometry (LC–MS/MS) analyses were used to isolate and identify merozoite antigens in the *T. equi* Florida strain that are recognized by antibody from horses infected with diverse isolates of *T. equi*. Post infection sera used in this study were collected from infected horses in Arizona, Georgia and Texas.

2. Materials and methods

2.1. Isolation of merozoites from horse erythrocytes infected with *T. equi*

Merozoites were isolated from erythrocytes of a horse clinically symptomatic persistently infected with the *T. equi* Florida strain (horse H3). Twenty-five mL of infected-*T. equi* erythrocytes with 49% parasitemia were centrifuged at 4000 × *g* for 15 min. The pellet was re-suspended in 10 mL of erythrocyte lysis buffer (Qiagen) and incubated for 5 min at room temperature (RT) in order to allow lysis of the erythrocytes. This was followed by centrifugation at 4000 × *g* for 15 min at 4 °C. The supernatant was removed and the pellet washed five times with 10 mL of cold phosphate buffer saline (PBS). Pelleted merozoites were re-suspended in 2 mL of proteinase inhibitor buffer (50 mM Tris pH8, 5 mM EDTA, 5 mM iodoacetamide, 1X proteinase cocktail [Roche]) with 1% NP-40 and stored at −20 °C.

2.2. Immunoblot detection of *T. equi* proteins that bind to polyclonal anti-*T. equi* equine antibodies

A whole lysate of *T. equi* merozoites proteins (prepared as described in Section 2.1) was separated by electrophoresis on a one dimensional (1D) pre-cast 4–12% SDS-PAGE Bis–Tris gel (NuPAGE®, Invitrogen) at 200 V for 50 min. After separation, proteins were transferred on to a nitrocellulose membrane and incubated with 10% skim milk diluted in PBS containing 0.05% tween 20 (blocking buffer) for 1 h at *RT*. The membrane was then incubated with sera from uninfected or infected *T. equi* horses diluted 1:50 in blocking buffer for 30 min at RT. The membranes were subsequently washed three times, 5 min each, with PBS containing 0.05% tween 20 (PBST). Following incubation with HRP-conjugated goat anti-horse IgG diluted 1:10,000 in blocking buffer, membranes were washed three times, 5 min each, with PBS containing 0.05% tween 20 (PBST). Chemiluminescent detection employed ECL™ Western blotting substrate (Amersham) for 5 min, and then membranes were exposed to ECL Hyperfilm™ (Amersham). Sera from ten different uninfected and *T. equi* clinically asymptomatic persistently infected horse sera were collected from horses in Arizona, Georgia and Texas were tested. Horse sera were previously tested by competitive ELISA (cELISA) and nested PCR (nPCR). Serum samples were tested in duplicate by cELISA according to the manufacturer's instructions (VMRD, Inc.). Nested PCR amplification was carried out on genomic DNA extracted from horse blood (Qiagen), and analyzed for *T. equi* using primers derived from the ema1 gene.

2.3. Identification of *T. equi* proteins using immunoaffinity chromatography and two-dimensional gel electrophoresis

Equine IgG was purified from serum of an horse experimentally infected with *T. equi* Florida strain (horse H5). Non-relevant protein often present in high abundance in serum was removed using the Melon™ Gel IgG Purification System (Pierce) according to manufacturer's instructions. After purification both concentration and quality of horse IgG were quantified using a NanoVue and visualized by SDS-PAGE and immunoblot, respectively. Briefly, proteins from *T. equi* merozoite lysates were separated by electrophoresis on a pre-cast 12% SDS-PAGE Bis–Tris gel (NuPAGE®, Invitrogen). After separation, proteins were transferred to a nitrocellulose membrane and incubated in blocking buffer for 1 h at RT. The membrane was incubated with purified horse IgG diluted 1:50 in blocking buffer for 30 min at RT, then washed three times, 5 min each, with PBST. Next, the membrane was incubated with HRP-conjugated goat anti-horse IgG diluted 1:10,000 in blocking buffer, washed three times and chemiluminescent detection was performed as described above.

To perform affinity-chromatography, purified equine IgG were dialyzed against coupling buffer (0.1 M $NaHCO_3$ pH 8.3 containing 0.5 M NaCl), and 9 mg of equine IgG was incubated overnight at 4 °C with 2 mL CNBr-activated Sepharose™ 4 Fast Flow beads (GE Healthcare) according to the manufacturer's instructions. Briefly, 2 mL CNBr-activated Sepharose™ 4 fast flow-coupled horse IgG antibodies were washed and packed in a glass column (10 mL). *T. equi* merozoite lysate was dialyzed against coupling buffer and antigens purifed using an ÄKTA™ avant system (GE Healthcare). Proteins were eluted at RT and 1 mL/min with a mix between buffer A (PBS, pH 7.4) and buffer B (0.1 M glycine, pH 2.5). Chromatographic conditions used were an isocratic run (100% A) for 15 min, followed by loading of the sample. Unbound material was then removed by washing with equilibration buffer for 20 min; subsequently a gradient elution was applied from 0 to 100% B for 2 min, an isocratic step at 100% B for 15 min, re-conditioning from 100% to 1% B for 1 min, followed by an isocratic step at 100% A for 10 min. Protein elution was detected by measuring the UV absorption at 280 nm and fractions were collected every 0.5 mL. Data collection and the sample chromatograms were acquired by using an ÄKTA™ avant system with UNICORN™ 6 control software (GE Healthcare).

Purified proteins were separated by 1D and two dimensional (2D) electrophoresis on a pre-cast 12% SDS-PAGE Bis–Tris gel. The 1D gel was performed as described in Section 2.2. For 2D gel analysis, *T. equi* proteins isolated by affinity chromatography were dissolved in a rehydration solution containing 1.1× ZOOM® 2D protein solubilizer 1, 1.6 mM DTT, 0.5% ZOOM® carrier ampholytes 3–10 (v/v) and 0.001% bromophenol blue according to the manufacturer's instructions (Invitrogen). The isoelectric focusing (IEF)