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Cocktail of *Theileria equi* antigens for detecting infection in equines

Shimaa Abd El-Salam El-Sayed[1,2#], Mohamed Abdo Rizk[1,3#], Mohamed Alaa Terkawi[1], Ahmed Mousa[4], El Said El Shirbini El Said[2], Gehad Elsayed[2], Mohamed Fouda[2], Naoaki Yokoyama[1], Ikuo Igarashi[1*]

[1]*National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-Cho, Obihiro, Hokkaido 080-8555, Japan*

[2]*Department of Biochemistry and Chemistry of Nutrition, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt*

[3]*Department of Internal Medicine and Infectious Diseases, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt*

[4]*Department of Biochemistry and Chemistry of Nutrition, Faculty of Veterinary Medicine, Sadat City University, Sadat City, Menoufyia, Egypt*

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ABSTRACT

Objective: To use two diagnostic antigens belonging to the frequently associated in *Theileria* domain, *Theileria equi* (*T. equi*) protein 82 (Te 82) and *T. equi* 104 kDa microneme-rhoptry antigen precursor (Te 43), to diagnose *T. equi* infection in horses as compared with equi merozoite antigen-2 (EMA-2).

Methods: In the current study, we applied a cocktail-ELISA containing two antigens (EMA-2 + Te 82) to diagnose *T. equi* infection either in experimentally infected horses or in field infection.

Results: Our findings have revealed that a cocktail formula of EMA-2 + Te 82 provided a more practical and sensitive diagnostic candidate for diagnosing *T. equi* infection in horses as compared with Te 82 or Te 43 alone.

Conclusions: The ELISA technique using a cocktail formula of EMA-2 + Te 82 offers a practical and sensitive diagnostic tool for diagnosing *T. equi* infection in horses and using of this promising cocktail formula will be applicable for epidemiological surveys and will help control the infection in horses.

1. Introduction

Equine piroplasmosis, a tick-borne parasitic disease that affects horses, mules, donkeys and zebras, is caused by *Theileria equi* (*T. equi*) and *Babesia caballi* and is typified by severe hemolysis that leads to hemoglobinuria, severe anemia, icterus and death [1]. The disease is associated with great economic loss due to the cost of treatment, loss of performance and restrictions in meeting international requirements related to exportation or participation in equestrian sports events [2]. Therefore, there is an urgent need to develop an effective diagnostic strategy for controlling the infection.

T. equi infection can be diagnosed directly by microscopic examination. However, detecting the parasite by microscopy is usually difficult during chronic infections because of low parasitemia, while serodiagnoses by immunofluorescent antibody test and ELISA are suitable for detecting antibodies in subclinical cases or in chronically infected animals with markedly low parasitemia [3,4].

In view of the absence of equine piroplasmosis in Japan and the increasing number of horses imported to the country from places where the infection is endemic, the development of a highly specific and sensitive diagnostic antigen for *T. equi* is urgently required. Equi merozoite antigen-2 (EMA-2) is a major surface protein of *T. equi* and is considered an important target for the development of an effective diagnostic reagent [5]. In addition, this antigen has highly antigenic properties and is recognized by ELISA in the sera of *T. equi*-infected animals worldwide [6]. However, further research on new antigens is extremely desirable for the development of a standard global diagnostic *T. equi* antigen.

*Corresponding author: Ikuo Igarashi, DVM, PhD, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-Cho, Obihiro, Hokkaido 080-8555, Japan.
Tel: +81 155 49 5641
Fax: +81 155 49 5643
E-mail: igarcpmi@obihiro.ac.jp

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[#]These authors contributed equally to this work.

Frequently associated in *Theileria* (FAINT) domains were first detected in *Theileria annulata* and *Theileria parva* as a stretch of 70 amino acids [7]. These domains were found to be overrepresented in proteins predicted to be secreted [7]. FAINT domain-containing genes are distributed throughout the *T. equi* genome, while they are not reported for *Babesia bovis* or *Plasmodium falciparum* [5]. In this study, two FAINT domains, including *T. equi* protein 82 (Te 82) and *T. equi* 104 kDa microneme-rhoptry antigen precursor (Te 43), were used as diagnostic antigens for *T. equi* infection in comparison with EMA-2. Te 82 was previously used to diagnose *T. equi* infection [8]; however, this is the first time that Te 43 protein has been established for the diagnosis of *T. equi* infection in horses.

Cocktail-ELISA is a recently developed diagnostic tool that possesses high diagnostic sensitivity [9]. Its previous use in the diagnosis of tuberculosis and schistosomiasis has resulted in improved specificities, positive predictive values and kappa values as compared with the use of a single antigen [9,10]. Although, this technique is highly sensitive in diagnosing infection, it has never been used for the diagnosis of *Babesia* and *Theileria* infection. Therefore, the aim of this study was to use cocktail-ELISA to diagnose *T. equi* infection using two diagnostic antigens (EMA-2 + Te 82). This study is considered the first to use cocktail-ELISA for diagnosing *T. equi* infection.

2. Materials and methods

2.1. Parasites

U.S. Department of Agriculture strains of *T. equi*, kindly provided previously by the Equine Research Institute of the Japan Racing Association, were grown in equine erythrocytes *in vitro*, as described by Avarzed *et al.*, using a microaerophilic, stationary-phase culture system [11]. Briefly, medium 199 for *T. equi* (Sigma–Aldrich, Tokyo, Japan) supplemented with 40% equine serum, 60 IU/mL of penicillin G, 60 μg/mL of streptomycin (Sigma) and 0.15 μg/mL of amphotericin B (Sigma) was used as a maintaining medium. Additionally, 13.6 μg/mL of hypoxanthine (ICN Biomedicals Inc., Costa Mesa, CA, USA) was added to the *T. equi* culture as a vital supplement. Cultures of parasitized red blood cells were incubated at 37 °C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. The cultured parasite was harvested when the parasitemia reached 8%–10%.

2.2. Cloning, expression and purification of the recombinant bacterial proteins

Six oligonucleotide primers (Table 1) were used to amplify the *EMA-2t*, *Te 82t* (23_Y–660_S aa) (accession number: XP_004831145) and *Te 43t* (20_I–389_C aa) (accession number: AFZ81467) genes from the cDNA by PCR [12]. The amplified DNA was digested with *EcoR* I and *Xho* I and then ligated into the *EcoR* I and *Xho* I sites of a pGEX-4T *Escherichia coli* (*E. coli*) expression plasmid vector (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom). The resulting plasmids, designated pGEX/EMA-2, pGEX/Te 82 and pGEX/Te 43, were used to transform into the *E. coli* DH5α strain and to express as glutathione *S*-transferase (GST). Fused bacterial proteins were purified from the soluble fraction with glutathione-sepharose 4B (GE Healthcare Bio-sciences AB, Björkgatan 30, Sweden).

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

The expressed recombinant proteins were verified by SDS-PAGE and boiled for 5 min in a sodium dodecyl sulfate sample buffer [62.5 mmol/L Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate, 5% mercaptoethanol, 10% glycerol, 0.02% bromophenol blue] and subjected to 12% SDS-PAGE (ATTO Corp., Tokyo, Japan); thereafter, the gel was stained with Coomassie blue stain for protein detection.

The *T. equi* proteins and control GST protein in SDS-PAGE gel were electrophoretically transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). For western blotting, the membrane was blocked with 0.05% Tween 20 with phosphate buffered saline (PBS-T) plus 3%–5% skimmed milk and probed with the positive *T. equi*-infected sera diluted at 1:500 for 1 h and then washed three times with PBS-T. The membrane was incubated with horseradish peroxidase-conjugated goat anti-horse immunoglobulin G (IgG) antibody (1:8 000 Bethyl) for 1 h. After three washes with PBS-T, reacted bands were visualized using a solution containing 3-diaminobenzidine tetrahydrochloride and H_2O_2 (Dojindo Molecular Technologies, Inc., Tokyo, Japan).

2.4. ELISAs

EMA-2, Te 82 and Te 43 proteins were used separately as antigens for ELISA or as a cocktail of EMA-2 + Te 82. These antigens were evaluated using a panel of the *T. equi* experimentally infected sera that had been collected serially from one horse during the course of infection. In addition to 44 serum samples from horses experimentally infected with *T. equi*, 376 field samples were collected from *T. equi* infection-endemic areas, including South Africa (262 samples) and Ghana (114 samples). The 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 50 μL of each recombinant protein at a concentration of 2 μg/mL per well in a

Table 1
Primers used in the study.

Genes	Primers	References
EMA-2t	5_-ACGAATTCTAAAATGTTGAGCAAG-3_ 5_-ACGAATTCCGATGAGGCACCAAAG-3_	[13]
Te 82t	5_-CGAATTCTATTTGCTGGATGTCCATGCA-3 5_-CCTCGAGTTATTTGTAACCATCATTCAA-3	This study
Te 43t	5_-CCGAATTCATAGAACCCAAAGAAGACAAA-3 5_-CCCTCGAGTTAACACATCTTTGGTGTGAT-3	This study

coating buffer (50 mmol/L carbonate-bicarbonate buffer, pH 9.6). The plates were washed once with 0.05% PBS-T and then incubated with 100 μL of a blocking solution (3% skim milk in PBS) for 1 h at 37 °C. After washing, the plates were incubated with 50 μL of the serum samples diluted 1:100 with the blocking solution for 1 h at 37 °C. The plates were washed six times with PBS-T and then incubated with 50 μL of horseradish peroxidase-conjugated goat anti-horse IgG antibody (Bethyl) diluted 1:4000 with the blocking solution for 1 h at 37 °C as a secondary antibody. The plates were washed six times as described above and 100 μL of a substrate solution [0.1 mol/L citric acid, 0.2 mol/L sodium phosphate, 0.3 mg/mL of 2, 2-azide-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) and 0.01% of 30% H_2O_2] was then added to each well. After incubation for 1 h at room temperature, the optical density (OD) was measured with an MTP-500 microplate reader (Corona Electric, Tokyo, Japan) at a wavelength of 415 nm. The ELISA result was determined for each sample by subtracting the mean OD value of two readings with GST protein from the mean OD value of two readings with the EMA-2, Te 43 and Te 82 proteins and a cocktail of EMA-2 + Te 82. Using receiver operating characteristic curve analysis with MedCalc statistical software (version 11.4), cutoff values were calculated on the basis of 25 non-infected equine sera.

3. Results

The genes encoding EMA-2, Te 43 and Te 82 were successfully expressed as soluble GST fusion proteins in *E. coli* with molecular masses of 50 kDa, 74 kDa and 85 kDa, respectively (Figure 1). Sera collected from horses experimentally infected with *T. equi* specifically reacted to all of the recombinant proteins but not to the control GST protein in western blot analysis, suggesting their high antigenicity in the infected sera (Figure 2). Furthermore, the specificity and sensitivity of EMA-2, Te 43 and Te 82 proteins and a cocktail of EMA-2 + Te 82 were evaluated in a standard ELISA with experimentally infected and negative-control equine sera and the cutoff OD values were determined to be 0.059, 0.088, 0.084 and 0.069, respectively (Figure 3).

The diagnostic performances of these recombinant proteins were evaluated with sequential sera from a horse infected with *T. equi* that had developed a high titer of antibody response to

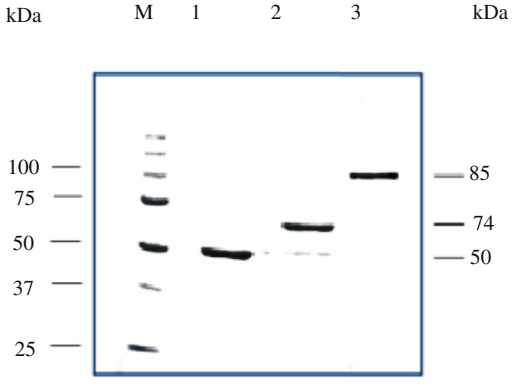

Figure 1. Successful expression of recombinant proteins in *E. coli*.
About 12% SDS-PAGE of recombinant protein stained with Coomassie blue. M: Molecular mass marker; Lane 1: Recombinant EMA-2; Lane 2: Recombinant Te 43; Lane 3: Recombinant Te 82. The size of each recombinant protein was indicated on the right.

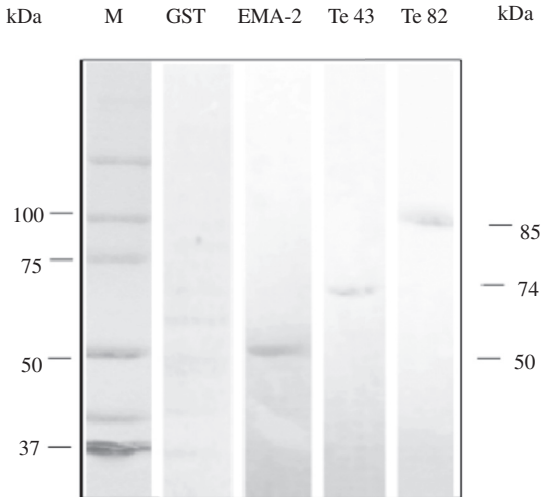

Figure 2. Western blot analysis with horseradish peroxidase-conjugated goat anti-horse IgG against different recombinant proteins.
GST, EMA-2, Te 43 and Te 82 were detected in the sera of *T. equi*. M: Molecular mass marker.

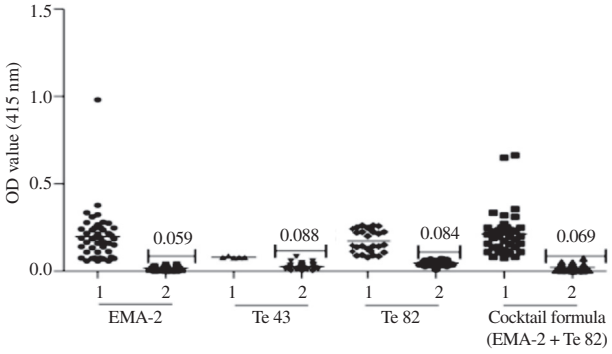

Figure 3. Reactivity of ELISA using recombinant proteins with equine sera.
Lane 1: Positive *T. equi*-infected sera; Lane 2: Negative sera samples. The cutoff value of each recombinant protein is indicated by a bar.

recombinant protein EMA-2 and a cocktail formula of antigens at Day 6 post infection, and this high antibody titer was maintained until 36 days post infection (Figure 4). Next, the sensitivity of the assay was evaluated using 44 serum samples from horses experimentally infected with *T. equi* and 376 field samples collected from endemic areas of *T. equi* infection, as previously mentioned. Of 44 experimentally infected serum samples, 28, 5 and 39 samples were found to be positive for Te 82, Te 43 and a cocktail formula of EMA-2 and Te 82,

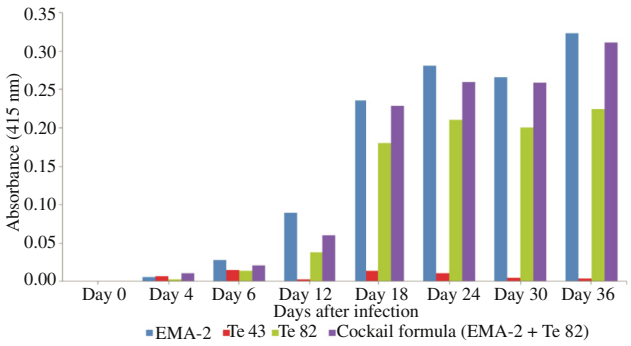

Figure 4. Reactivity of ELISA using recombinant proteins with sequential sera from horses experimentally infected with *T. equi*.

respectively (Table 2). While, for the field samples, 224, 213, 9 and 220 sera samples showed higher OD than cutoff values for EMA-2, Te 82, Te 43 and a cocktail formula of EMA-2 and Te 82, respectively (Table 3). The fewer number of positive samples detected by Te 43 indicated the low potential of this protein as a diagnostic antigen. The specificity and sensitivity of ELISAs with serum samples were determined on the basis of EMA-2 results. The specificity results were 79.87%, 96.90% and 90.00%, while the sensitivity results were 79.35%, 13.63% and 83.48% for Te 82, Te 43 and a cocktail of (EMA-2 + Te 82), respectively (Table 4). Additionally, a cocktail formula (EMA-2 + Te 82) showed the highest kappa value among the antigens used (Table 4). These results revealed the high diagnostic performance of the cocktail formula for diagnosing *T. equi* infection in horses.

Table 2
Summary of ELISA results with recombinant proteins with serum samples from horses experimentally infected with *T. equi*.

Antigens	Positive samples	Negative samples
EMA-2	44	–
Te 82	28	16
Te 43	5	39
Cocktail formula (EMA-2 + Te 82)	39	5
Be 82[a]	8	5

[a] : Data were from Hirata *et al*. [8]. Be 82: *Babesia equi* 82.

Table 3
Summary of ELISA results with recombinant proteins with 376 field sera collected from *T. equi*-endemic areas, South Africa and Ghana.

Antigens	Positive samples	Negative samples
EMA-2	224	152
Te 82	213	163
Te 43	9	367
Cocktail formula (EMA-2 + Te 82)	220	156

Table 4
Specificity, sensitivity and kappa value of ELISA.

Antigens	Sensitivity (%)	Specificity (%)	Kappa
Te 82	79.35	79.87	0.58
Te 43	13.63	96.90	0.08
Cocktail formula (EMA-2 + Te 82)	83.48	90.00	0.74
Be 82/1-235[a]	31.00	100.00	ND
EMA-2[b]	97.00	96.00	0.93

[a] : Data were from Hirata *et al*. [14]. [b] : Data were from Kumar *et al*. [6]; ND: Not detected.

4. Discussion

Equine piroplasmosis is considered a serious threat due to the international movement of infected horses [13]. Therefore, developing an effective diagnostic candidate for such infections is urgently required. Serological tests, particularly ELISA, seem to be the most practical and economical technique for epidemiological investigation [15]. Indeed, as compared to other serological tests, the ELISA technique offers greater sensitivity, objectivity and capacity for rapid adaptation in examining a large number of serum samples. The crude antigen prepared from merozoites has traditionally been utilized for the serological detection of parasite antibodies. However, recombinant protein can be an alternative source, allowing better standardization of the test [9].

The high antigenicity of the EMA-2 antigen for detecting *T. equi* infection might be attributable to the fact that EMA-2 is a unique protein that was not expressed during any of the asexual erythrocytic developmental stages of *T. equi* merozoites but was expressed only during the early developmental stage [13]. These findings indicate that the expression of EMA-2 on the merozoite surface is highly stage-specific. Additionally, the EMA-2 antigen was mutually expressed on the surface of extra-erythrocytic merozoites, and the intra-erythrocytic merozoites shed only EMA-2 on the erythrocytic cytoplasm and/or inside membrane surface, which is responsible for its erythrocytic binding behavior [13].

Our results reveal the good diagnostic performance of the Te 82 antigen for detecting the infection with high specificity and sensitivity values. The Te 82 antigen used in this study exhibited higher sensitivity and lower specificity values than those obtained previously with the Be 82/1-235 antigen [14]. Moreover, the Te 82 antigen used in this study has the ability to detect infection 12 days earlier than Be 82 [8], confirming the good diagnostic performance of this antigen for detecting *T. equi* infection.

Although our findings revealed that the cocktail formula of EMA-2 and Te 82 antigens demonstrated the same performance as EMA-2 alone, the cocktail formula has several advantages over the single antigens.

The cocktail formula requires less volume of antigens than single antigens do. Moreover, until now, no single recombinant ELISA containing a single antigen has been able to detect all IgG- or immunoglobulin M-positive samples examined across different stages of the disease. There are different theories as to why this might be. First, the humoral immune response varies with the stage of infection [16]. Hence, some antibodies present at one stage of infection may be absent in other stages and vice versa. This requires that multiple epitopes from different antigens are present in an immunoassay to detect the antibodies present at different disease stages. Second, the ability to reconstitute native epitopes in recombinant proteins produced in *E. coli* is dependent on the expression vector and the protein purification employed. Therefore, combining two or more recombinant proteins will increase the sensitivity of recombinant-ELISAs, as has been previously determined for toxoplasmosis [17], tuberculosis and schistosomiasis, resulting in improving specificities, positive predictive values and kappa values as compared with using a single antigen [9,10].

In conclusion, our study has revealed that the ELISA technique using a cocktail formula of EMA-2 + Te 82 provides a new, practical and sensitive diagnostic tool for diagnosing *T. equi* infection in horses and helps better manage such disease conditions. A cocktail-ELISA of different antigens might help improve the diagnostic strategy of equine *Babesia* and *Theileria* parasites.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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