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Diagnosis of *Theilria equi* Infections in Equines Using Immunoaffinity Purified Antigen

[1]*Mona S. Mahmoud,* [2]*Amr E. El-Hakim,* [1]*Seham H.M. Hendawy,*
[1]*Hatem A. Shalaby,* [1]*Omnia M. Kandil and* [1]*Nadia M.T. Abu El-Ezz*

[1]Parasitology and Animal Diseases Department, National Research Centre
[2]Molecular Biology Department, National Research Centre,
33 El Bohouth St., Dokki, 12622, Giza, Egypt

Abstract: *Theileria equi* (*T. equi*) and *Babesia caballi*(*B. caballi*) are obligate intra-erythrocytic parasites of equines. It is not possible to differentiate between *T. equi* and *B. caballi* infections on the basis of clinical signs. Thus, in many instances, it has been pointed out that serological and molecular techniques represent a more objective tool for the diagnosis of equine piroplasmosis. The low specificity of enzyme-linked immunosorbent assay (ELISA) in detection of *T. equi* antibodies in infected equines motivated this study to isolate and identify antigenic proteins in the *T. equi* Egyptian strain that were recognized by antibodies from donkey infected with *T. equi* using affinity chromatography. The diagnostic efficacy of the *T. equi* affinity purified antigen (APA) was evaluated using ELISA and Western blotting. The purification improved the diagnostic potency of the antigen as the obtained results using *T. equi* APA was higher than the crude antigen results and the best concentration of the serum was 1:400 in the diagnosis of *T. equi* to overcome the phenomenon of cross-reactivity between *T. equi* and *B. caballi*. In turn, the study was extended to detect specific antibodies to *T. equi* in sera of apparently healthy horses and donkeys. The ELISA results showed that the percentage of *T. equi* infection was 26% and it was higher in donkeys (30%) than in horses (22%). By Western blot technique, two polypeptides of molecular weights 190 and 30 KDa were considered to be common cross reactive bands between *T. equi* and *B. caballi* antigens and were not recognized in *T. equi* APA. At the same time, *T. equi* APA revealed three antigenically active polypeptides at molecular weights of 100, 37 and 20 KDa that were not recognized by *B. caballi* serum. Those polypeptides might be reinforced the specificity of *T. equi* APA to be higher than that of *T. equi* crude antigen in detection of anti-*T. equi* antibodies. The data present in this work are relevant for the development of improved diagnostic method that allows us to gain insight into the almost unexplored epidemiological situation of equine piroplasmosis and apply effective control measures.

Key words: Equine · *Theilria equi* · ELISA · Western Blot · Diagnosis

INTRODUCTION

Equine piroplasmosis, an infectious tick-borne disease of Equidae, is an Office International des Epizooties (OIE) list disease. It is caused by two intra-erythrocytic haemoprotozoan *Babesia equi* (*B. equi*) and *B. caballi* in most tropical and subtropical areas of the world, as well as in some temperate zones [1]. Based on clarification of the life cycle and analysis of its full genome, it was recognized that the originally named *B. equi* apicomplexan more closely resembles *Theileria* species and it has thus been renamed *T. equi* [2, 3]. *B. caballi* invades erythrocytes only while, *B. equi* is also capable of infecting lymphocytes [4]. *T. equi* is considered a more virulent species than *B. caballi* [2]. Persistent infection by either parasite restricts the international movement of horses. The introduction of carrier animals into areas where tick vectors are prevalent can lead to an epizootic spread of the disease. Equine babesiosis has posed a threat to the international movement of horses [5] because horses from a *babesia* free zone, when introduced into an endemic area, suffer clinically from the disease resulting into death of large number of animals. Previous data collected in Egypt,

Corresponding Author: Mona, S. Mahmoud, Department of Parasitology and Animal Diseases, National Research Centre, P.O. Box 12622, Giza, Egypt. E-mail: monasaid3000@yahoo.com.

suggest high prevalence of equine piroplasmosis which is consistent with the current lack of control measures [6-9]. However, the overall impact of piroplasmosis on the equine populations in Egypt remains unknown, and thus more studies using sensitive and specific diagnostic techniques are still required. Direct demonstration of parasites in blood smears by microscopic examination is an accurate but insensitive form of detection, especially in the persistent phase of infection. Reliable, sensitive, and specific tests for equine piroplasmosis are therefore very important not only for disease control but also for the prevention of introduction of parasites into countries that are regarded as free of the infection/disease. Serological methods, such as complement fixation test (CFT) [10, 11], the card test [12] and the indirect fluorescent antibody test (IFA) [13, 14] have been used for diagnosis of *B. equi* infection. The CFT yields a considerable number of false-negative results [15, 16]. The IFAT is laborious and not amenable to standardization. The advantages of the ELISA in contrast to CFT or IFAT are its high sensitivity and the possibility of standardization and computer evaluation. However, there is a need for increasing specificity of the ELISA by using species specific antigens. Indeed, in this study, affinity chromatography with immobilized IgG antibodies against *T. equi* were used to isolate and identify antigenic proteins in the *T. equi* Egyptian strain that were recognized by antibody from donkey infected with *T. equi*. The diagnostic efficacy of the affinity purified *T. equi* antigen was evaluated using ELISA and Western blotting.

MATERIALS AND METHODS

Parasite Isolation: The *T. equi* Egyptian strain, confirmed by nested PCR, was isolated from infected donkey and used as a source of antigenic material for production of crude and purified antigens.

Splenectomy of Infected Donkey: *T. equi* infected donkey with parasiteamia > 2% was splenectomized. The spleen was exteriorized following the technique of El-Zomor [17].

Nested PCR: Total DNA was extracted; using FTA® Elute cards (Whatman Cat. No.WB120410) following the manufacturer's instructions, from the blood sample of infected donkeys (positive in microscopic examination and in *T. equi*). The PCR was performed using 5-10 µl eluted DNA in a final volume of 25 µl containing 12.5 µl Jump Start RED Taq Ready Mix PCR reaction mix (Sigma-Aldrich), 10 pmol of each primer [18]. The oligonucleotide primer sequences are shown in Table 1. For PCR amplification, DNA was initially denatured at 95 °C for 3 min, followed by 25 cycles, consisting of denaturation at 95 °C for 15 sec, annealing at 60 °C for 15 sec and extension at 72 °C for 15 sec. A final extension cycle at 72 °C for 5 min was performed and reactions were cooled to 15 °C. Positive controls, kindly denoted from the OIE Equine piroplasmosis reference lab located in Pullman, WA, and negative control were included for PCR amplifications. The amplifications for the primary PCR for *T. equi* were performed in a thermocycler (Techne-Cyclogene). Amplified DNA samples were electro-phoresed on 1.5% agarose gel and stained with Sybr Safe (Invitrogen). The length of the amplified products was estimated using a 100 bp DNA ladder and the amplified products were visualized with an UV trans-illuminator (Bachofer D7410) and photographed using gel Documentation system (BioDocAnalyze-Biometra Analytic GmbH). The specific amplification products of *T. equi* and *B. caballi* were detected at 229 and 222 bp, respectively.

Sequencing of PCR Products: The PCR products were purified for sequencing using the QIA quick Spin PCR Purification kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. Sequencing reactions were performed using oligonucleotide primers that were used in the PCR. Sequencing of the PCR products was performed by the GATC Company with an ABI 3730xl DNA sequencer, using the nested forward and reverse primers. Each sequencing reaction was repeated three times in both the forward and reverse directions before being accepted for analysis. Sequences derived from Babesia sp. were assembled using ChromasPro 1.49 beta (Technelysium Pty. Ltd., Tewantin, QLD, Australia).

Sampling

Blood Samples: Blood samples from about 50 horses from police academy and 50 donkeys from the zoo were available for analysis. This study was conducted in Egypt in the area selected for the clinical study. Two blood samples per animal were required in this study (one without anticoagulant for serology and another containing EDTA for detecting parasiteamia).

Table 1: Oligonucleotide primer pairs used in PCR amplifications for the detection of *Babesia* species in equines.

Parasite	Primer name	PCR reaction	Gene name	Primer sequence
T. (B.) equi	Beq-F	External	Equi Merozoite Antigen -1 gene (ema-1 gene)	5'-GAG GAG GAG AAA CCC AAG-3'
	Beq-R			5'-GCC ATC GCC CTT GTA GAG-3'
	BeqN-F	Nested		5'-TCA AGG ACA ACA AGC CAT AC-3'
	BeqN-R			5'-TTG CCT GGA GCC TTG AAG-3'
B. caballi	Bca-F	External	B. caballiRhoptry associated protein gene (RAP-1 gene)	5'-GATTACTTGTCGGCTGTGTCT-3'
	Bca-R			5'-CGCAAGTTCTCAATGTCAG-3'
	BcaN-F	Nested		5'-GCTAAGTACCAACCGCTGA-3'
	BcaN-R			5'-CGCAAGTTCTCAATGTCAG-3'

Serum Samples:

- Serum sample from *T. equi* infected donkey, Egyptian strain, was obtained during the course of infection (Positive control).
- *B. caballi* positive serum as well as the negative control sera were kindly denoted from USDA lab Pullman, USA.
- Sera from apparently healthy horses and donkeys.

All sera were stored at-20 °C until analyzed.

Preparation of *T. equi* Crude Antigen: The antigen was prepared according to Goodger *et al*. [19] and Commins *et al*. [20]. Blood of *T. equi* infected splenectomized donkey was collected on 10% EDTA at the peak of parasitaemia (15%) centrifuged at 1500 rpm for 10 min. and resuspended in 0.15 M PBS pH 7.2 for 3 times. Packed erythrocytes were mixed with cold distilled water at ratio of 1:3. The suspension was recentrifuged at 10.000 rpm for 50 min. at 4 ºC. The supernatant lysate crude antigen was removed, the protein content was measured by the method of Lowry *et al*. [21] and stored at -20 ºC.

Preparation of Affinity Purified Antigen (APA): The *T. equi* antigen was purified using two successive chromatographic steps; including Protein G and Cyanogen Bromide Sepharose 4B affinity columns, which kindly denoted from USDA lab Pullman, USA.

Purification of Serum Immunoglobulins (IgGs): Serum sample immunoglobulins (IgGs) obtained from donkeys infected with *T. equi* (Egyptian strain) were purified by affinity chromatography using protein G-Sepharose CL-4B column (1.6×4 cm) according to the instructions of the manufacturer. The unbound proteins were washed with 20 mM PBS pH 7.2, and the bound proteins were eluted with 0.1 M glycine-HCl, pH 2.7. Two ml fractions were collected at a flow rate of 48 ml/hr, and the absorbance of each fraction was measured at 280 nm.

Cyanogen Bromide Sepharose 4B Affinity Column: The purified immunoglobulins (IgGs) of the donkey anti- *T. equi* antigens were dialyzed against coupling buffer (0.1M NaHCO3, 0.5 M NaCl, pH 8.4) and coupled to Cyanogen bromide-activated Sepharose 4B (CNBr-Sepharose) as recommended by Pharmacia Fine Chemicals (Sweden). The excess reactive sites were blocked by blocking buffer (0.1M Tris-HCl, pH 9.0). The prepared column was equilibrated with 20 mM phosphate-buffer (PB), pH 7.4, prior to use. *T. equi* antigens were equilibrated with equilibrating buffer and loaded on the CNBr-Sepharose coupled to the IgGs column at a flow rate of 20 ml/h. The unbound proteins were washed with the equilibration buffer at a flow rate of 30 ml/h. The bound proteins were eluted with 0.1 M glycine-HCl, pH 2.5, into Tris-base in order to restore the pH to 7.2. The eluted proteins were dialyzed against 20 mMTris-HCl, pH 7.4, and 0.5 M NaCl, and were designated as affinity-purified antigen (APA).

Protein Determination: Protein content of the different samples was determined according to Lowry method [21].

Indirect ELISA for Detection of *T. equi* Antibodies: *T. equi* antibodies were detected in equine sera using crude and APA according to Avarzed *et al*. [22]. Briefly, ELISA plates were coated overnight at 4 °C with 100 µl of 10 µg/well of either *T. equi, B. caballi* (obtained from USDA lab Pullman, USA), crude antigens or APA of *T. equi* in 50 mM carbonate buffer, pH 9.6. After blocking with 1% skim milk in coating buffer, and washing with 0.01 M PBS/0.05 % Tween, pH 7.4, serial dilutions from 1:100 to 1:800 of normal and positive horse sera in washing buffer were added and incubated for 1 hr at 37 °C.

The plates were washed and incubated with 1:1000 dilution of anti-horse IgG peroxidase conjugate (Sigma), diluted 1:1000, for 1 hr. The wells were then washed and 100 µl of O-phenylenediamine solution (0.33 mg/ml in citrate buffer, pH 5.2, in the presence of 0.04 % hydrogen peroxide) were added. The reaction was stopped after 10-15 min by the addition of 20 µl of a 1:20 dilution of sulfuric acid. The tested serum samples were diluted at 1:400. A serum sample was considered positive for antibody to *T. equi* if it showed an OD higher than the mean plus (3x standard deviations) of negative serum samples. Reading of the optical density (OD) was at 405 nm using an ELISA plate reader (ELx800 UV, BIO-TEK Instruments).

Sodium Dodecyle Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting: *T. equi*, *B. caballi* crude antigens and *T. equi* APA were electrophoresed in 10% polyacrylamide gels according to Laemmli [23], the proteins were electrophoretically transferred from the gels onto nitrocellulose membrane for 60 min at 100 V with a wet transfer cell [24]. The antigenic and cross-reactive proteins were recognized by using of *T. equi* positive serum, *B. caballi* positive serum and negative serum.

RESULTS

Isolation of *T. equi* Isolate: One day before splenectomy the parasitaemia was 2% and the parasitaemia reach its peak (15%) at the third day after splenectomy then begin to decrease again. The infected blood (parasiteamia 15%) was used for antigen preparation.

Nested PCR and Sequencing: The nested PCR using total DNA extracted from the blood of donkey, which was previously assigned as *T.equi* positive, based on microscopic examination and nPCR, confirmed that we have *T.(B.) equi* pure strain (Fig 3A and B).

Sequence analysis on two sequenced 231bp nPCR amplicons produced using *T. (B) equi* specific primers demonstrated 100% identity to the *Babesia equi* merozoite antigen-1 reference sequence (GenBank Accession number AF261824).

Purification of Donkey Anti-*T. equi* Immunoglobulins: The IgGs of donkey infected with *T. equi* were purified using protein G-Sepharose. The donkey's serum was separated into unbound proteins and bound proteins, which were mainly anti-*T. equi* IgGs (Fig. 1).

Preparation of *T. equi* APA: The purified IgGs were coupled to CNBr-Sepharose and used to purify the *T. equi* immunogens. About Ten mg of total *T. equi* natural proteins were loaded on the CNBr-IgG column. The chromatographic profile of *T. equi* antigens on the CNBr-Sepharose coupled to IgGs showed the separation of the antigens into unbound proteins and bound immunogens (Fig. 2) which were used in serolological studies (ELISA).

ELISA Results: The results in Figure 3 (a, b and c), testing the ability of the three selected antigens (*T. equi* lysate, *T. equi* APA and *B. caballi* lysate) in binding with specific antibodies of *T. equi* positive, *B. caballi* positive and negative sera revealed that cross-reaction between the two crude antigens and sera remained even with increasing serum dilutions. At the same time, *T. equi* APA reacted specifically with its corresponding sera after 1:200 dilution. The purification improved the diagnostic potency of the antigen as the obtained results using *T. equi* APA was higher than the lysate antigen results. The results demonstrated that the best dilution of serum was 1:400 in the diagnosis of *T. equi* to overcome the phenomenon of cross-reactivity between *T. equi* and *B. caballi*. In turn, at the selected serum dilution, the presence of antibodies against *T. equi* was determined in serum samples from 50 horses and 50 donkeys using *T. equi* APA with ELISA. A total number of 26 out of 100 serum samples (26%) were found positive for *T. equi* antibodies. The prevalence of infection was higher in donkeys (30%) than in horses (22%) as shown in Table 2.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE): Electrophoretic separation of *T. equi* lysate, APA, *B. caballi* lysate showed multiple fractions at both high and low molecular weight ranges (Fig. 4). The electrophoretic profile of *T. equi* lysate gave 8 polypeptides with different molecular weights (195, 142, 60, 54, 31, 27, 23 and 19 kDa). While, *T. equi* APA gave 5 polypeptides at molecular weights of 100, 70, 44, 37 and 27 kDa. The electrophoretic profile of *B. caballi* lysate gave 9 polypeptides with different molecular weights at 193, 155, 128, 109, 87, 68, 42, 27, and 19 kDa. A Common band was detected at 27 kDa for the three antigens.

Western Blot: To clarify specificity of the recorded babesial polypeptides, the adopted western blot technique (Fig. 5) revealed that antigenically active components in *T. equi* lysate, on reaction with its homologous sera, were three polypeptides at molecular weights of 190, 90 and 30 Kda (Fig.5A, Lane 1).

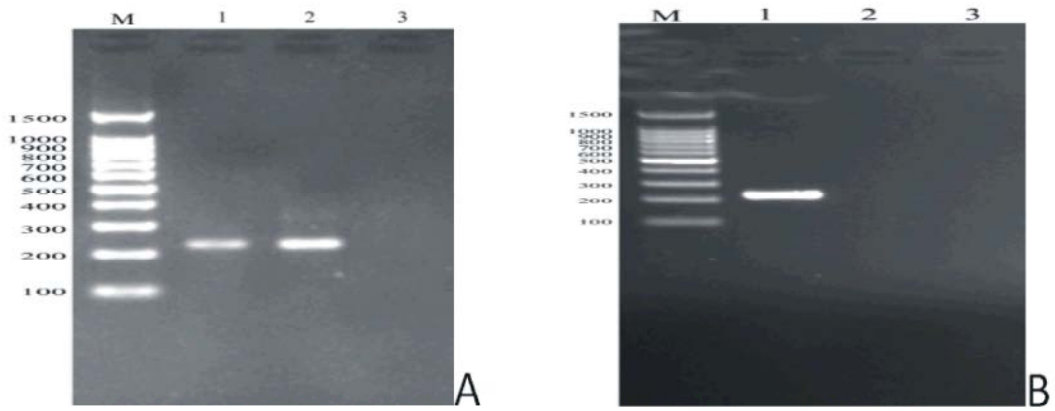

Fig. 3: The amplified PCR products were electrophoresed on agarose gels, stained with SybrSafe, and visualized under UV light, and densitometry images were recorded. Marker (M) consisting of a 1-kb DNA ladder were included for each gel.
A. nPCR amplification of a 229 bp amplicon using T.(B.) equi EMA-1 specific primers on purified T.(B.) equi DNA (lane 1); Identical PCR amplifications performed on experimental tested sample (lane 2), Negative control (lane 3).
B. nPCR amplification of a 229 bp amplicon using B. caballi Bc48 specific primers on purified B. caballi DNA (lane 1); Identical PCR amplifications performed on experimental tested sample (lane 2), Negative control (lane 5).

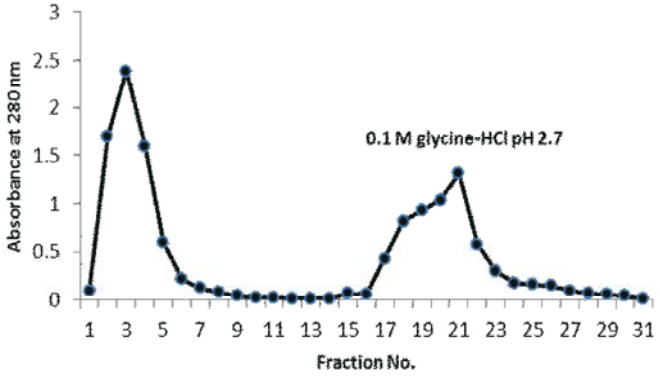

Fig. 1: Affinity chromatography of donkey anti- *T.equi* on protein G- Sepharose 4B column (1.6 × 4 cm)

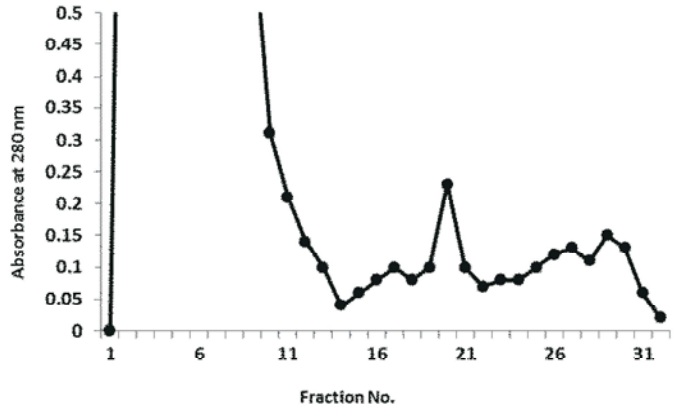

Fig. 2: Affinity chromatography of T. equi lysate proteins on CNBr-activated Sepharose 4B coupled to T. equi IgG (1.6 × 5 cm).

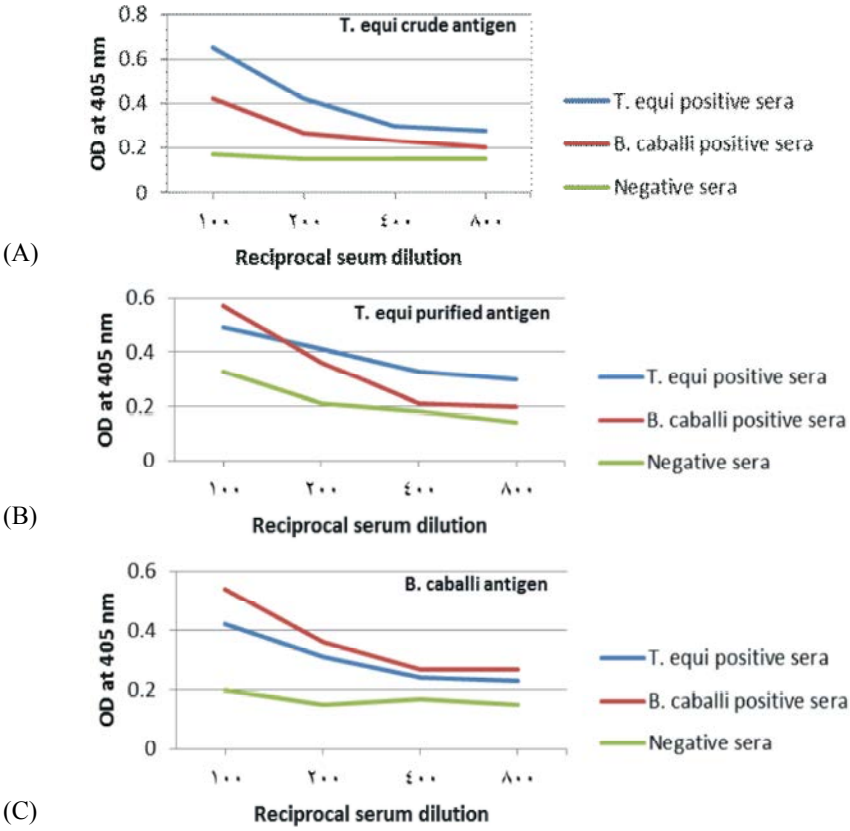

Fig. 3: Reactivity of different Babesia antigen preparations in ELISA with T. equi positive, B. caballi positive and negative horse sera.(a) T. equi crude antigen, (b) T. equi purified antigen, (c) B. caballi crude antigen

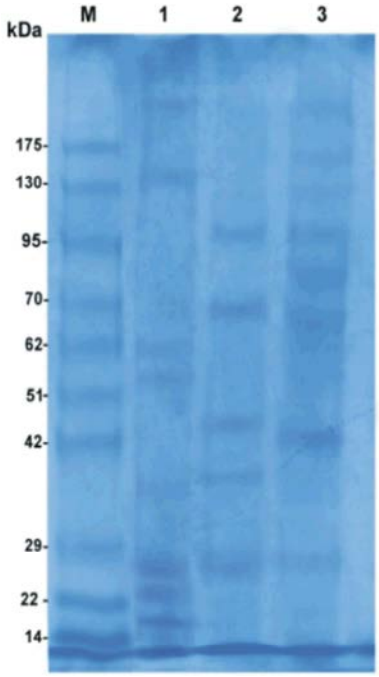

Fig. 4: The electrophoretic profile of *T. equi* lysate (Lane 1), *T. equi* APA (Lane 2) and *B. caballi* lysate (Lane 3). M: Molecular weight standard

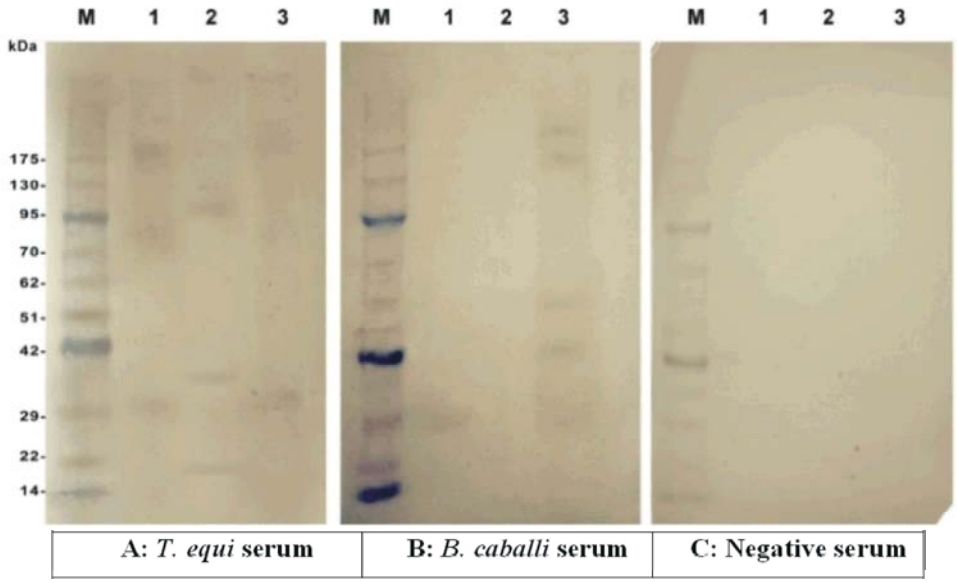

Fig. 5: Recognition of different Babesia antigens by homologous and heterologous sera using Western blot. Lane 1: *T. equi* lysate, Lane 2: *T. equi* APA, Lane 3: *B. caballi* lysate. M: Molecular weight Standard

Table 2: Detection of *T. equi* antibodies in serum samples of horses and donkeys as determined by indirect ELISA using affinity purified antigen.

Type of equine	Location	No. of tested animals	No. of positive	Infection (%)
Horses	Police Academy	50	11	22
Donkeys	Zoo garden	50	15	30
Total		100	26	26

While, *B. caballi* serum reacted crossly with two polypeptides at molecular weights of 190 and 30 KDa (Fig.5B, Lane 1). At the same time, *T. equi* APA revealed three antigenically active polypeptides at molecular weights of 100, 37 and 20 KDa (Fig.5A, Lane 2). All those polypeptides were not recognized by *B. caballi* serum (Fig.5B, Lane 2). Concerning *B. caballi* lysate, two polypeptides at molecular weights of 190 and 30 KDa out of five antigenically active polypeptides recognized by its homologous serum (Fig.5A&B, Lane 3) showed cross reactivity toward *T. equi* serum. No polypeptides were identified by negative sera (Fig.5C). Thus, both polypeptides of molecular weights 190 and 30 KDa were considered to be common cross reactive bands between *T. equi* and *B. cabali* antigens. At a time, those bands were not recognized in *T. equi* APA.

DISCUSSION

It is extremely difficult to diagnose the piroplasma organisms in carrier animals by means of the microscopic examination of blood smears. The serological testing of animals is recommended as a preferred method of diagnosis, especially when horses are destined to be imported into countries where the disease does not occur, but the vector is present. The enzyme-linked immunosorbentassay (ELISA) may be an alternative for increased and sensitive detection of acute and latent babesial infections, but its development has been hindered by a limited antigen supply and poor specificity [25]. Götz [26] demonstrated that sera positive for *B. equi* cross-reacted with *B. caballi* and *B. rodhaini* antigens. Merkle [27] reported the cross-reactivity of *B. caballi* serum with *B. equi* antigen and Aicher [28] showed cross-reactions between both species. Peymann *et al.* [29] and Böse and Peymann[30] reported the heightened sensitivity of ELISA when detergent-treated *B. caballi*-infected erythrocytes were used as antigen. However, cross-reactions were observed with 20% of the *B. equi* immune sera tested. The problem of low specificity was attributed to the use of crude antigens in the tests, mainly erythrocytes infected with the equine babesias or crude whole parasites. To increase the specificity of the ELISA, developments and optimization had been directed towards the identification of species specific antigens. In the current study, affinity chromatography with equine postinfection antibody was used to isolate merozoite antigen in the *T. equi* Egyptian strain that was recognized by antibody from horses infected with diverse isolates of *T. equi*. Indeed, two extensively studied *T. equi* merozoite

surface proteins, belonging to the *T. equi* merozoite antigens (EMA) family (EMA-1 and EMA-2) were known to elicit antibody during *T. equi* infection in horses [31]. Both, EMA-1 and EMA-2 proteins had been identified as immunodominant antigens [32] that share 52% amino acid identity. In this study, the purification improved the diagnostic potency of the antigen as the obtained results using *T. equi* APA was higher than the lysate results and the best concentration of the serum was 1:400 in the diagnosis of *T. equi* to overcome the phenomenon of cross-reactivity between *T. equi* and *B. caballi*. In turn, the study was extended to detect specific antibodies to *T. equi* in sera of apparently healthy horses and donkeys. The ELISA results showed that the percentage of *T. equi* infection was 26% and it was higher in donkeys (30%) than in horses (22%). These results closely related with the IFA results, with its high sensitivity and specificity, of the previous study with USA as the total percentage was 26.6% and the infection rate in horses and donkeys was 23.9% and 31.4%, respectively (data not published). In Egypt, Ahmed [6] used the IFA test and found the infection rate was 93.88, 91.43 and 86.00% in donkeys, horses and mules, respectively. Avarzed *et al*. [22] succeeded by applying IFAT and ELISA using partially purified antigens and it was 97.4% for detection of antibody to *B. equi*. Asenzo *et al.* [33] developed an indirect ELISA for the detection of specific anti-*T. equi* antibodies in horse serum.. In Sudan, Salim *et al.* [34] found that 4.4% were positive for *B. caballi* and 63.5% were positive for *T. equi* by using the purified recombinant proteins (*B. caballi*48-kDa and the *T. equi*EMA-2) as antigens in the ELISA test.

By Western blot technique, two polypeptides of molecular weights 190 and 30 Kda were considered to be common cross reactive bands between *T. equi* and *B. caballi* antigens and were not recognized in *T. equi* APA. At the same time, *T. equi* APA revealed three antigenically active polypeptides at molecular weights of 100, 37 and 20 KDa that were not recognized by *B. caballi* serum. Those polypeptides might be reinforced the specificity of *T. equi* APA to be higher than that of *T. equi* crude antigen in detection of anti-*T. equi* antibodies as observed in ELISA test. Several authors had described the identification of babesial proteins that might be of diagnostic use. Knowles *et al.* [35] used equine erythrocytes infected with the USDA strain of *B. equi* and sera of naturally and artificially infected horses. Eleven major proteins ranging from 210-25 kDa were identified in immuno-precipitated preparations which had been separated by electrophoresis under reducing conditions. Ali *et al.* [36] used *B. equi* (USDA strain) merozoites separated by Percoll density gradient centrifugation. After electrophoresis under reducing conditions, the proteins were immunoblotted with immune sera to *B. equi*. A range of proteins from 96-15 kDa was detected which were species specific. Bose and Hentrich [37] identified nine major antigens of MW 41-19 kDa in *B. equi* (USDA) strain infected erythrocytes by Western blotting, of which only four were recognized by sera from field and experimentally infected European horses. A protein of MW 34 kDa of partially purified merozoites of *B. equi* (OP strain) was recognized by immune sera from naturally and artificially infected horses from different countries after Western blotting. This protein reacted specifically in indirect ELISA after purification from SDS-PAGE gels by excising and electroelution [38]. A lysate of *B. equi*-infected erythrocytes (USDA strain) was separated by two-dimensional electrophoresis and analysed by Western blotting. Nine major antigens or antigen groups with molecular weight ranging from 43 to 19 kDa were recognized by sera from horses experimentally infected with the USDA strain. Thus, four antigens with molecular weight of 33, 31, 19 and 20 kDa were identified as diagnostic antigens for European isolates of *B. equi*. None of the antigens diagnostic for European isolates was recognized by sera from field-infected horses from Brazil [37].

The present study demonstrated the higher specificity of *T. equi* APA than *T. equi* crude antigen in detection of anti-*T. equi* antibodies. Those observations were extended by demonstrating the value of the *T. equi* APA in detection of anti-*T. equi* antibodies in apparently healthy horses and donkeys. It provided evidence of shared antigens between *T. equi* and *B. caballi* antigens as well as specific positive reactivity of antigenically active polypeptides of *T. equi* APA. The results in this study are relevant for the development of improved diagnostic method that allows us to gain insight into the almost unexplored epidemiological situation of equine piroplasmosis and apply effective control measures.

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REFERENCES

1. Steinman, A., T. Zimmerman, E. Klement, I.M. Lensky, D. Berlin, Y. Gottlieb and G. Baneth, 2012. Demographic and environmental risk factors for infection by *Theileria equi* in 590 horses in Israel Vet. Parasitol., 187: 558-562.
2. Mehlhorn, H. and E. Schein, 1998. Redescription of *Babesia equi* Laveran, 1901 as *Theileria equi*. Parasitol. Res., 84: 467-475.
3. Kappmeyer, L.S., M. Thiagarajan, D.R. Herndon, J.D. Ramsay, E. Caler, A. Djikeng, J.J. Gillespie, A.O. Lau, E.H. Roalson, J.C. Silva, M.G. Silva, C.E. Suarez, M.W. Ueti, V.M. Nene, R.H. Mealey, D.P. Knowles and K.A Brayton, 2012. Comparative genomic analysis and phylogenetic position of *Theileria equi*. BMC Genomics., 13: 306.
4. Schein, E., G.G. Rehbein, W.P. Voigt and E. Zweygarth, 1981. *Babesia equi* (Laveran 1901) 1. Development in horses and in lymphocyte culture. Zeitschrifr fir Tropenmedizin und Parasitologie, 32: 223-227.
5. Friedhoff, K.T., A.M. Tenter and I. Muller, 1990. Haemoparasites of equines: impact on international trade of horses. Rev. Sci. Tech., 9: 1187-1194.
6. Ahmed, Z.G., 1981. Studies on the different species of *Babesia* infecting equine by fluorescent antibody. M.V.Sc. Thesis (Parasitology), Fac. Vet. Med. Cairo, Univ.
7. Abdel-Hady, S.M.R., 1984. Biological studies on *Babesia equi* and a comparative study on the different treatment. M.V. Sc Thesis (Parasitology), Fac. Vet. Med. Cairo, Univ.
8. Rampersad, J., E. Cesar, M.D. Campbell, M. Samlal and D. Ammons, 2003. A field PCR for the routine detection of *Babesia equi* in horses. J. Vet. Parasitol., 114: 81-87.
9. Farah, A.W., N.A. Hegazy, M.M. Romany, Y.A. Soliman and A.M Daoud, 2003. Molecular detection of *Babesia equi* in infected and carrier horses by polymerase chain reaction. Egypt. J. Immunol., 10: 793-799.
10. Donnelly, J., L.P. Phipps and K.L. Watkins, 1982. Evidence of maternal antibodies to *Babesia equi* and *B. caballi* in foals of seropositive mares. Equine Vet. J., 14: 126-128.
11. Weiland, V.G., B.M. Aicher and J. Boch, 1984. Serodiagnostic und Therapienkontrolle der Pferdepiroplasmosemit KBR und IFAT. Berl. Munch. Tierarztl. Wochenschr., 97: 341-439.
12. Amerault, T.E., W.M. Frerichs and D. Stiller, 1979. Comparative serologic study of equine piroplasmosis, with card and complement fixation tests. Am. J. Vet. Res., 40: 529-531.
13. Callow, L.L., W. McGregor, B. Rodwell, R. Rogers, G. Fraser, D. Mahoney and G. Robertson, 1979. Evaluation of an indirect fluorescent antibody test to diagnose *Babesia equi* infection in horses. Aust. Vet. J., 55: 555-559.
14. Donnelly, J., L. Joyner, O. Graham-Jones and C. Ellis, 1980. A comparison of the complement fixation and fluorescent antibody tests in a survey of the prevalence of *Babesia equi* and *Babesia caballi* in horses in the Sultanate of Oman. Trop. Anim. Health Prod., 12: 50-60.
15. Weiland, G., 1986. Species-specific serodiagnosis of equine piroplasma infections by means of complement fixation test (CFT), immunofluorescence (IIF), and enzyme-linked immunosorbent assay (ELISA). Vet. Parasitol., 20: 43-48.
16. Tenter, M. and K.T. Friedhoff, 1986. Serodiagnosis of experimental and natural *Babesia equi* and *B. caballi* infections. Vet. Parasitol., 20: 49-61.
17. El-Zomor, S.T., 1985. Comparative studies on splenectomy procedures in certain farm animals. S. Sc. thesis, Cairo University, Egypt.
18. Baptista, C., Lopes, M.S. Tavares, A.C. Rojer, H. Kappmeyer, L. Mendonca, and D.A. da Camara Machado, 2012. Diagnosis of *Theileria equi* infections in horses in the Azores using cELISA and nested PCR. Ticks and Tick-borne diseases, 4: 242-245.
19. Goodger, B.V., I.C. Wright and D.J. Waltisbuhl, 1983. The lysate from erythrocytes infected with *Babesia bovis*. Z. Parasitenked., 69: 473-482.
20. Commins, M.A., B.V. Goodger and I.G. Wright, 1985. Proteinases in the lysate of bovine erythrocytes infected with *Babesia bovis*: Initial vaccination studies. Int. J. Parasitol., 15: 491-495.
21. Lowry, D.H., N.J. Rosenbrough, A.L. Far and R.J. Randal, 1951. Protein measurement with folin phenol reagent. J. Biol. Chem., 193: 265-275.
22. Avarzed, A., D.T. de Waal, I. Igarashi, A. Saito, T. Oyamada, Y. Toyoda and N. Suzuki, 1997. Prevalence of equine piroplasmosis in central Mongolia. Onderstepoort J. Vet. Res., 64: 141-145.
23. Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

24. Towbin, H., T. Staehelin and J. Gordon, 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Nat. Acad. Sci. USA., 176: 4350-4354.
25. Bruning, A., 1996. Equine piroplasmosis an update on diagnosis, treatment and prevention. Br. Vet. J., 152: 139-151.
26. Götz, F., 1982. Untersuchulagenüber die Brauchbarkeit yon ELISA, IFAT, IHA und KBR zum Nachweis yon *Babesia equi* Infektionen, Dissertation. Veterinary Faculty, University of Munich.
27. Merkle, F.J., 1983. Umersuchungen fiber die Brauchbarkeit von KBR, IFAT und ELISA zumNachweis der *Babesia caballi*-Infektion des Pferdes. Dissertation. Veterinary Faculty, University of Munich.
28. Aicher, B.M., 1984. Möglichkeiten der serologischenDifferenzierung der *Babesia equi* und *Babesia caballi*-Infektion des Pferdesmit Hilfe yon KBR, IFAT, und ELISA. Dissertation. Veterinary Faculty, University of Munich.
29. Peymann, B., R. Böse and K.T. Fredhoff, 1993. Development of an ELISA and Western blot for the diagnosis of *Babesia caballi* infections in horses and comparison with immunofluorescence antibody test (IFAT) and complement fixation test (CFT). In 14th international Conference of the World Association for the Advancement of Veterinary Parasitology,Cambridge, UK.
30. Böse, R. and B. Peymann, 1994. Diagnosis of *Babesiacaballi* in fections in horses by enzyme linked immunosorbent assay and Western blot. Int. J. Parasitol., 24: 341-346.
31. Knowles, D.P., L.S. Kappmeyer and L.E. Perryman 1997. Genetic and biochemical analysis of erythrocyte-stage surface antigens belonging to a family of highly conserved proteins of *Babesia equi* and *Theileria* species. Mol. Biochem. Parasitol., 90: 69-79.
32. Knowles, Jr.D.P., L.S. Kappmeyer, D. Stiller, S.G. Hennager and L.E. Perryman, 1992. Antibody to a recombinant merozoite protein epitope identifies horses infected with *Babesia equi*. J. Clin. Microbiol., 30: 3122-3126.
33. Asenzo, G., S. Wilkowsky, M. Barrandeguy, M. Mesplet, D. Benitez and M. Florin-Christensenc, 2008. Development of an Indirect ELISA for the Diagnosis of Equine Piroplasmosis. Ann. N.Y. Acad. Sci., 1149: 235-238.
34. Salim, B.O.M., S.M. Hassan, M.A. Bakheit, A. Alhassan, I. Igarashi, P. Karanis and M.B. Abdelrahman, 2008. Diagnosis of *Babesia caballi* and *Theileria equi* infections in horses in Sudan using ELISA and PCR. Parasitol. Res., 103: 1145-1150.
35. Knowles, D.P.Jr., L.E. Perryman, L.S. Kappmeyer and S.G. Hennager, 1991. Detection of equine antibody to *Babesia equi* merozoite proteins by a monoclonal antibody-based competitive inhibition enzyme-linked immunosorbent assay. J. Clin. Microbiol., 29: 2956-2958.
36. Ali, S., C. Sugimoto, M. Matsuda, T. Sugiura, T. Kanemaru, M. Onuma and M. Kamada, 1993. Protein characterization of *Babesia equi* piroplasms isolated from infected horse erythrocytes. Parasitol. Res., 79: 639-643.
37. Böse, R.I. and B. Hentrich, 1994. Identification of antigens diagnostic for European isolates of *Babesia equi* by two-dimensional electrophoresis and western blotting. Parasitol. Res., 80: 182-185.
38. Bruning, A., 1994. Equine piroplasmosis an update on diagnosis, treatment and prevention. Br. Vet. J., 152: 139.