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FULL PAPER

Diagnostic application of recombinant equine merozoite surface antigen-1 in elisa for detection of *Theileria equi* specific antibodies

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Abstract

Theileria equi **merozoite surface antigens have been an important candidate for development of diagnostics. We developed ELISA based on EMA-1 recombinant antigen, so as to widen our diagnostic confidence in detection of antibodies against *T. equi* in sero-surveillance studies. The 547 bp EMA-1 gene fragment encoding high hydrophilic antigenic region was expressed with glutathione-S-transferase tag in prokaryotic system and purified protein (43 kDa) was used for development of ELISA (EMA-1t/ELISA). The EMA-1t/ELISA clearly differentiated *T. equi*-infected from *Babesia caballi*-infected horse sera or normal horse sera. The results of the study were validated with previously developed $_{EMA\text{-}2}$ELISA on serum samples of known *T. equi* infection status and a very high correlation (0.93) was recorded between the relative percent positivity (RPP) values. Further diagnostic sensitivity of EMA-1t/ELISA was 0.92 while specificity was 1.0, indicating its suitability for sero-epidemiological studies. This assay was applied on serum samples ($n = 240$) collected from field horses in northern part of India. High sero-prevalence of *T. equi* antibodies were diagnosed in serum samples collected from Haryana state (74%) and Uttarakhand state (36.31%). Results of this study suggested that the 43 kDa EMA-1 expressed protein could be a reliable immunodiagnostic antigen in ELISA for *T. equi* sero-prevalence studies.**

Key Words: *Theileria equi*, EMA-1, diagnosis, ELISA, *Babesia equi*.

Introduction

Equine piroplasmosis is a tick-borne protozoan disease of horses, mules, donkeys and zebra, caused by an intra-erythrocytic apicomplexan protozoa *Theileria equi* and/or *Babesia caballi*. This disease is of major economic significance as the affected animals manifest decreased working

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capacity, in-appetence, malaise, and chronic weight loss. Equine piroplasmosis is a major stumbling block in international movement of the infected horses[9,10,16]. Infections by *T. equi* have wider geographic distribution than *B. caballi* and mostly synchronize with the presence of tick-vectors[5,18].

Theileria equi infection is endemic in India and responsible for appreciable economic losses to equine owners. Horses, infected with *T. equi*, remain carriers for the life and act as source of infection for ticks, which in turn act as vectors of the disease transmission[18]. Ticks of *Hyalomma* species are the potential biological vector for transmission of disease among equines[23]. *Theileria equi* infection in equines can be diagnosed by number of different methods including Giemsa-stained blood smears, *in vitro* culture method, ELISA and PCR. Microscopic demonstration of intra-erythrocytic parasite is still the best, most reliable, economical and sustainable method for confirmative diagnosis of clinical equine babesiosis, but not suitable for detection of latently infected equids[8]. Therefore, serological tests such as enzyme linked immunosorbent assay (ELISA), indirect fluorescent antibody test (IFAT), have been developed and being recommended by OIE as official tests for international transport of equids[26].

Most of the diagnostics developed for diagnosis of Babesiosis and Theileriosis in bovines and equines have utilized parasitic immunodominant antigen[2,27,20,13,17]. Equine merozoite surface antigens (EMA-1 and EMA-2) are immunodominant proteins of *T. equi*.[14,19]. It has been demonstrated that EMA-1 and EMA-2 are not expressed during all the erythrocytic-developmental stages of *T. equi* merozoites and that these two antigens are co-expressed during the early developmental stages[19]. Further phylogenetic analysis has also shown high genetic diversity of EMA-1 gene among different strains of *T. equi*[20]. Previously we developed EMA-2 recombinant antigen based ELISA for detection of *T. equi* specific antibodies. EMA-1 has been considered an important diagnostic target[4,30,15] for diagnosis of *T. equi* antibodies. In this study we intended to develop ELISA using Indian strain specific EMA-1 recombinant antigen of *T. equi* and to examine the comparative sensitivity and specificity of these two assays, so that further recommendations could be applied for diagnostic purpose on Indian equine population.

Materials and Methods

The Parasite cDNA: Whole blood was collected in EDTA from a *T. equi* latently infected pony (being maintained in large animal house facility of National Research Centre on Equines, Hisar) and red blood cells (RBCs) were isolated. The RBCs lysate was processed for RNA extraction and purification as per RNeasy Protect Animal Blood Kit (Qiagen, India) protocol. The RNA obtained was quantified on a photometer (Biophotometer™ plus, Eppendorf, Germany) and stored at −40°C for further use. cDNA was synthesized form the above eluted RNA using commercial kit (Enhanced Avian RT First Strand Synthesis Kit -STR-1, Sigma). This cDNA fragment was used in PCR amplification and further expression studies.

Protein antigenic determinants and PCR primer: A method developed by Hopp and Woods[12] was used for locating the antigenic determinants in EMA-1 amino acid sequences with the help of Laser gene Protean software [version 7.1.0, DNASTAR Inc.]. The antigenic determinants were analysed and accordingly primer pair was designed so as to amplify 547 bp gene fragment of EMA-1 (accession no KC347576) using online primer blast programme of NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast). The following primers were used for amplification of the desired product in PCR and processed further for gene expression: forward 5-GAATTCACGTCGTCTACACTGCCCA CGA-3; reverse 5-GAATTCTCAGCGGCATCCTT GACCTCAA-3; (the underlined letters in the primers represent the *Eco*RI cloning site).

Expression of EMA-1 protein and its purification: For expressions of truncated EMA-1 (accession number: KC347576) gene products in *E. coli*, plasmid vectors was constructed[28]. Briefly, this truncated version of EMA-1 was without N-terminal signal peptides and C-terminal hydrophobic trans-membrane domain. EMA-1 gene was amplified from the *T. equi* cDNA template by PCR using above primer set. A 547-bp long truncated EMA-1 gene was amplified in PCR and processed further for *Eco*RI digestion, purification (gel cleaned) and ligated into cloning site of pGEX-4T *E. coli* expression vector (Amersham Pharmacia Biotech, Piscataway, NJ). The resultant plasmid (pGEX/EMA-1t-547/p), was used to express the glutathione *S*-transferase (GST) tagged gene product, which was purified on glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) as described previously[3,28].

SDS-PAGE and Western Blot Analysis: The purified EMA-1 recombinant protein (EMA-1t/547) was mixed with equal volume of 2X SDS sample buffer [62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 5% 2-mercaptoethanol, 2% SDS, and 0.01% bromophenol blue]. The material was heated at 95°C for 5 min, followed by centrifugation at 8000 *g* for 10 min. The supernatant was collected and loaded on to wells of 10% poly-acrylamide gel (SDS-PAGE) and subjected to electrophoresis at a constant current of 60 mA for 60 min[24] until bromophenol blue dye front reached above the bottom edge of the resolving gel. The proteins in the gel were stained/destained or transferred (semi-dry electrophoretic transfer) on to polyvinylidene fluoride (PVDF) membrane (Hybond-P, GE Healthcare Japan Corporation, Tokyo, Japan) at 0.8 mA/cm^2 and processed for immunoblotting as described previously[29]. The PVDF membranes were incubated overnight at 4°C with blocking buffer [3% bovine serum albumin (BSA) in PBS, pH 7.0]. Thereafter, the membranes were washed three times with a washing buffer [50 mM Tris-HCl (pH 7.0), 150 mM NaCl, and 0.05% (v/v) Tween 20] and incubated with serum collected from *T. equi* latently infected pony (diluted to 1 : 20 in 1% BSA in PBS) for 1 h. After washing, the membrane was incubated with rabbit anti-horse IgG (whole molecule) antibody conjugated with horseradish peroxidase (HRP) (1 : 4000; cat no.: A6917, Sigma Aldrich, India) for 1h and, finally, the membrane was incubated with substrate solution containing diaminobenzidine (DAB, 0.5 mg/ml) and 0.03% H_2O_2 to develop and visualize the specific immunogenic peptide bands on the blotted membrane. Molecular weights of the individual bands were determined by comparing them with the respective fraction on the stained SDS-PAGE gel.

Enzyme linked immunosorbent assay using EMA-1t/547 *(EMA-1t/ELISA):* The optimum dilution of recombinant protein (EMA-1t/547), conjugate (rabbit anti-horse IgG antibody conjugated with HRP, Sigma Aldrich) and known positive reference serum were determined using a chequer-board titration. Each well of ELISA plate (Greiner Bio-one, Germany) was coated with 50 µl of recombinant protein (EMA-1t/547) diluted in 0.1 M carbonate/bicarbonate buffer (pH 9.6). The antigen coated ELISA plate was incubated overnight at 4°C. The un-adsorbed antigenic sites were blocked by incubating the ELISA plate with 3% BSA in PBS pH 7.0 (3% BSA-PBS) followed by four washings with PBS containing 0.05% Tween 20 (PBS-T). The test serum samples collected from field and reference serum samples were diluted to 1 : 200 in 1% BSA-PBS and 50 µl volume of each sample was added to the wells of the ELISA plate in duplicate. ELISA plate was incubated at room temperature (RT) for 1 h followed by four washings with PBS-T. Rabbit anti-horse IgG antibody conjugated with HRP was diluted to appropriate dilution with 1% BSA-PBS and 50 µl volume and transferred to each well of ELISA plate. ELISA plate was incubated at RT for 1 h followed by four washings with PBS-T. 100 µl of substrate solution [3 mg O-phenylene diamine-dihydrochloride (OPD, Sigma

Aldrich) powder was dissolved in 10 ml of phosphate citrate buffer (0.1 M citric acid, 0.2 M sodium Phosphate) and 3 µl of H_2O_2 was added] was added to each well of the ELISA plate. The ELISA plate was covered with aluminium foil and incubated for 5–7 min at RT, till the development of orange-brown colour. Once the colour developed, the reaction was stopped by adding 50 µl 3 M H_2SO_4. The absorbance in the wells of the ELISA plate was read at 492 nm (OD_{492}) by ELISA plate reader (BioTek, USA). The mean OD_{492} of negative control wells was calculated. The ELISA optimized and developed in this study was designated as EMA-1t/ELISA.

Determination of cut-off threshold value: For calculation of cut-off threshold value, 53 serum samples were selected from a previous study[20]. A total 120 serum samples had been collected from different organized stud farms and tested for the presence/absence of *T. equi* antibodies by applying $_{EMA\text{-}2}$ELISA and cELISA[20]. These 53 serum samples had been tested negative by these two assays and were chosen for determining cut-off threshold RPP value in EMA-1t/ELISA.

The OD_{492} value of these serum samples in EMA-1t/ELISA were used for determining relative per cent positivity (RPP) as described by Kumar *et al*,[21]. The cut off RPP value for considering the positive reaction of a sample in EMA-1t/ELISA was selected by calculating mean RPP of 53 serum samples (as above) + 4 × standard deviation (SD). A serum sample showing RPP value above cut-off was considered serologically positive.

Reference serum samples: Experimental donkeys had been infected with *T. equi* parasite and sequential serum samples were collected[22]. *T. equi* infection positive ($n = 23$) serum samples were selected from these experimentally infected animals after appearance of *T. equi* parasites in blood smears. *T. equi* infection negative samples ($n = 13$) were also selected from control animals which had been found negative for *T. equi* parasite by repeated blood smear examination and for absence of *T. equi* specific antibodies in $_{EMA\text{-}2}$ELISA.

A reference *B. caballi* positive serum was obtained from United State Department of Agriculture (USDA), USA (received as part of the CFT kit). The serum samples positive for *Trypanosoma evansi*, glanders, strangles, equine infectious anaemia and equine influenza were obtained from different laboratories at NRCE, Hisar.

EMA-1t/ELISA validation, diagnostic sensitivity, specificity and cross-reactivity: These *T. equi* positive and negative reference serum samples were tested separately by EMA-1t/ELISA and $_{EMA\text{-}2}$ELISA and individual RPP values were calculated. The diagnostic sensitivity and specificity of the EMA-1t/ELISA was evaluated against $_{EMA\text{-}2}$ELISA on these serum samples[20,22]. Similarly cross-reactivity of EMA-1t/ELISA with horse serum positive for *B. caballi*, *Trypanosoma evansi*, glanders, strangles, equine infectious anaemia and equine influenza was also tested.

Field serum samples: Serum samples were collected randomly from 240 field horses reared some geographic localities of Haryana ($n = 50$) and Uttrakhand ($n = 190$) states of India. These serum samples were subjected for diagnosis of *T. equi* specific antibodies using EMA-1t/ELISA as developed in the present study.

Results

Protein antigenic determinants and PCR primer

The hydrophobic region in N-terminal (1-21aa) and C-terminal (234-271aa) of EMA-1 peptide were not considered while designing the sense and anti-sense primers. The primer pair was so designed as to amplify and express truncated EMA-1 gene from 52 to 233 protein codons yielding 182 amino acids long peptide.

T. equi recombinant protein (pGEX/EMA-1t-547)

The PCR amplified product (547 bp) was sequenced so as to confirm the desired orientation of the gene (data not shown). The recombinant plasmid clone expressed as 43 kDa GST fused protein (pGEX/EMA-1t-547), as confirmed on SDS-PAGE, which immune-reacted with The *T. equi* reference positive serum (Fig. 1).

EMA-1t/ELISA and cut-off threshold value

The optimum dilutions of EMA-1t/547 recombinant antigen, the conjugate and test serum sample were determined as 1.8 µg/well (36 ng/µl), 1 : 10000 and 1 : 200, respectively. All the 53 serum samples collected from non-endemic organized horse stud farms showed EMA-1t/ELISA OD_{492} ranging from 0.23 to 0.30. The mean RPP and SD were calculated as 7.06% and ±3.77, respectively. The cut off RPP value for the positivity of test sample in EMA-1t/ELISA = $7.06+4 \times 3.77 = 22.17$. Hence, RPP value of >22% was considered as cut off threshold for determining presence of *T. equi* antibodies in the tested serum sample.

EMA-1t/ELISA validation, diagnostic sensitivity, specificity and cross-reactivity

A very high correlation ($r^2 = 0.93$) was observed between the RPP values as calculated on *T. equi* infection positive ($n = 23$) and negative ($n = 13$) serum samples by EMA-1t/ELISA and $_{EMA\text{-}2}$ELISA (Fig. 2). The diagnostic sensitivity of $_{EMA\text{-}2}$ELISA and EMA-1t/ELISA was 0.95 and 0.92, respectively, while diagnostic specificity was 1.0 with both the assays. All the *T. equi* negative infection status samples were detected negative in both of the assay and hence diagnostic specificity was 1.00.

The serum samples positive for *T. evansi*, glanders, strangles, equine infectious anaemia and equine influenza did not show any cross-reactivity (>1 : 200 dilution) in EMA-1t/ELISA and OD_{492} values were much the same as observed with *T. equi* reference negative serum samples and RPP values were between −12.97 to −27.42, indicating non cross-reactivity of EMA-1t/ELISA.

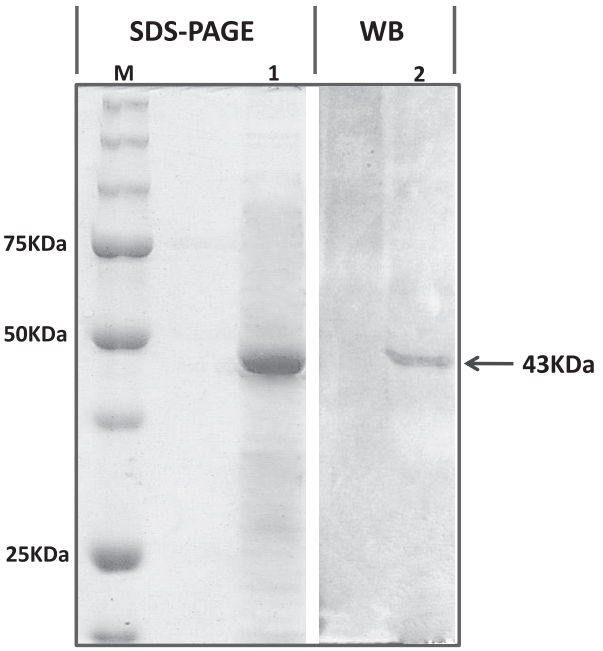


Fig. 1. Expression and purification of the GST tagged EMA-1 (truncated) protein (pGEX/EMA-1-1t-547) on SDS-PAGE (lane 1) and its immune-reaction with *T. equi* positive serum sample on immunoblot analysis (lane 2). The purified pGEX/EMA-1-1t-547 protein was of 43 kDa. WB: Western blot.

Sero-prevalence of T. equi antibody

The field samples collected from Haryana area showed high prevalence of *T. equi* specific antibodies as compared to samples collected from Uttarakhand area. Out of 240 serum samples tested in EMA-1t/ELISA, 74% samples (from Haryana area) were found positive to *T. equi* antibodies, while 36.31% samples from Uttarakhand area were positive in EMA-1t/ELISA.

Discussion

Antigenic differences in EMA-1 and EMA-2 protein sequences have been demonstrated among different geographical isolates of *T. equi*[1,11,20]. Therefore, there was an increasing need to compare the sensitivity and specificity of

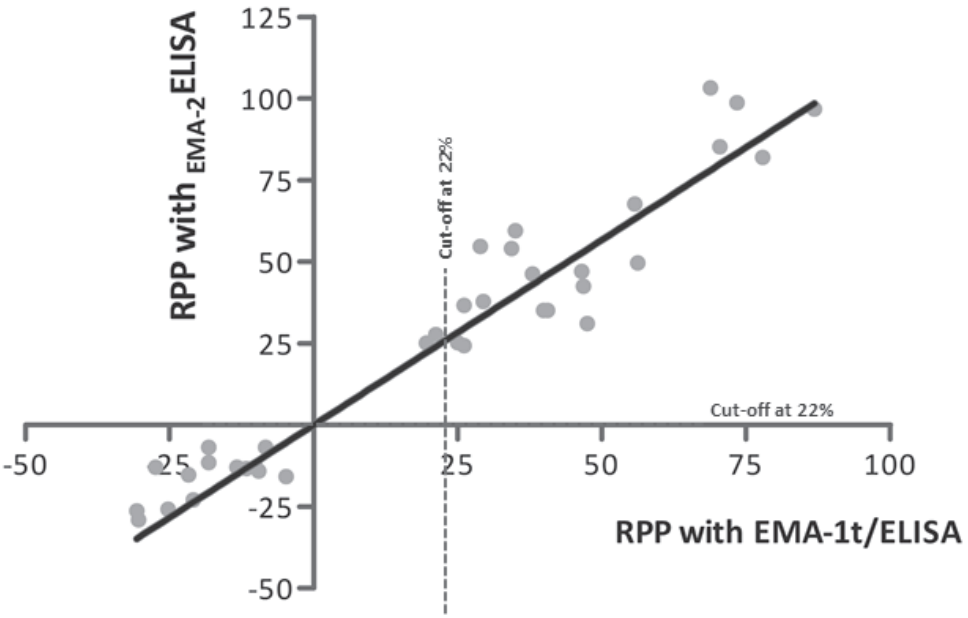


Fig. 2. Validation of RPP value as obtained in EMA-1t/ELISA and $_{EMA-2}$ELISA on serum samples of *Theileria equi* known infection status. Scattered graph with linear line between corresponding RPP values spot showed very high degree of correlation (0.93) indicating absolute correlation.

diagnostics developed on these recombinant antigens, so as to address its applicability on a geographical region. In continuation of our previous study, we intended to develop another ELISA based on EMA-1 recombinant antigen (EMA-1t/ELISA), so as to widen our diagnostic confidence in detecting *T*. equi specific antibody among equine population.

Hydrophilicity plot and antigenic index of EMA-1 protein was used to determine the peptide segment to be expressed as recombinant protein. The EMA-1 is a 271 amino acid (AA) long peptide and we expressed 182 AA long peptide spanning from 52 - 233AA in the EMA-1 protein (accession no. AGL76728). The truncated expressed protein (182AA) has low hydrophobicity and more charged amino acid as compared to complete EMA-1 protein. The hydrophobic residues in a protein preferentially tend to remain in the core, while hydrophilic residues occur at the outer surface of the folded protein and hence make the protein more antigenic and reactive[25]. A 43 kDa GST-tagged EMA-1 recombinant protein was obtained (EMA-1t/547), reacting distinctly with anti-*T. equi* horse serum (Fig. 1), while GST protein (tag protein only) did not show any immune reaction with *T. equi* positive serum (data not shown), indicating high specificity of the expressed recombinant protein.

RPP methodology was adopted for establishing cut-off threshold value in EMA-1t/ELISA, as it helped in better differentiation between *T. equi* antibody positive and negative OD and has been applied by many workers[6,7,21]. There was a very high correlation (0.93) between the RPP values observed by the two assays ($_{EMA-2}$ELISA and EMA-1t/ELISA; Fig. 2) on serum samples of known *T. equi* infection status. This has validated the results obtained by EMA-1t/ELISA developed in this study. Further diagnostic sensitivity of EMA-1t/ELISA was also comparable to ELISA based on EMA-2 recombinant antigen ($_{EMA-2}$ELISA). There were a few samples which were *T. equi* infection positive but detected negative in $_{EMA-2}$ELISA (one samples) and EMA-1t/ELISA (two sample). These samples represented early phase of *T. equi* infection in experimentally infected donkeys (collected during 12–18 days PI) and antibody titre might have been low. Diagnostic specificity of this assay was 100%,

indicating accuracy of the diagnostic in identifying true - *T. equi* - negative samples.

T. equi sero-positivity was observed in samples collected from Haryana state then from Uttrakhand area. Haryana state falls in trans-gangetic plains region, while Uttrakhand is a part of upper-gangetic plains region, due to which there is appreciable climatic differences affecting the tick-vector distribution. Kumar *et al*[20] also recorded the same observations while applying $_{EMA-2}$ELISA on samples collected from these regions. These sero-positive horses were apparently healthy and not showing any symptoms of the disease condition.

It can be concluded from this study that EMA-1 recombinant protein-based ELISA (EMA-1t/ELISA) was able to detect specific antibodies against *T. equi* parasite in equine serum samples with high sensitivity and specificity and can be applied on field sero-surveillance studies.

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Conflict of interest statement

The authors of this research paper declare that they have no conflict of interest.

Author Contributions Statement

Conceived and designed the experiments: Sanjay Kumar. Performed the experiments: Sandeep Kumar, Liza Goyal, Ashok Kumar, Rajender Kumar and Sanjay Kumar. Analyzed the data: Naresh Rakha, Parveen Goel and Sanjay Kumar. Contributed reagents/materials/analysis tools: Sanjay Kumar and Rajender Kumar. Wrote the paper: Sanjay Kumar.

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