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Development of EMA-2 recombinant antigen based enzyme-linked immunosorbent assay for seroprevalence studies of *Theileria equi* infection in Indian equine population

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ABSTRACT

Equine piroplasmosis is a tick-transmitted protozoan disease caused by *Theileria equi* and/or *Babesia caballi*. In the present study, we expressed a 53 kDa protein from the truncated EMA-2 gene of *T. equi* (Indian strain) and developed $_{\text{EMA-2}}$ELISA using this expressed protein. This ELISA is able to detect *T. equi*-specific antibodies in experimentally infected animals as early as 9 days post-infection. The assay developed was validated with the OIE recommended competitive ELISA (cELISA) on 120 serum samples and significant agreement (kappa = 0.93) was observed between results of both the ELISAs which indicates suitability of $_{\text{EMA-2}}$ELISA for use in sero-diagnosis. Diagnostic sensitivity and specificity of $_{\text{EMA-2}}$ELISA – as compared with cELISA – were 0.97 and 0.96, respectively. Analysis of 5651 equine serum samples – collected during 2007–2012 from 12 states of India representing eight agro-climatic zones – by $_{\text{EMA-2}}$ELISA revealed 32.65% seroprevalence of *T. equi* in India. In conclusion, the $_{\text{EMA-2}}$ELISA developed using the *T. equi* EMA-2 recombinant protein as antigen for detecting *T. equi*-specific antibodies has good diagnostic potential for sero-epidemiological surveys.

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1. Introduction

Equine piroplasmosis is an important disease of equines which is 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 capacity, in-appetence, malaise, and chronic weight loss. Equine piroplasmosis is a major stumbling block in international movement of the infected horses (Friedhoff et al., 1990; Hailat et al., 1997; Knowles, 1996). These parasites are widely distributed throughout the world (Asian continent, Europe, Africa, South America) and prevalence corresponds with the presence of the tick-vectors (Kumar and Kumar, 2007; De Waal, 1992). Equine piroplasmosis caused by *T. equi* is endemic in India (Kumar et al., 1997, 2003, 2013; Malhotra et al., 1978) and frequent clinical cases have been reported (Sharma et al., 1982; Sharma and Gautam, 1977). Outbreaks of this disease have been experienced in India whenever naive animals are introduced into endemic zones (Gautam and Dwivedi, 1976). Such situation has been evidenced by re-emergence of *T. equi* infection in Texas, United States (Ueti et al., 2012).

Asia is a habitat for about 30% of the world's equine population. *T. equi* infection is endemic in many regions as more than 35% latent infections has been reported from various countries *viz.*, India (Kumar and Kumar, 2007); Pakistan (Rashid et al., 2009); Mongolia (Munkhjargal et al., 2013); and China (Xu et al., 2003; Chahan et al., 2006). Development of sensitive and specific immunodiagnostics which can detect all the stages of the disease has been one of the

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primary research aims for equine piroplasmosis. Two types of immunodominant merozoite surface proteins *viz.*, equi merozoite antigen (EMA)-1 (34 kDa) and EMA-2 (30 kDa) have been identified in *T. equi* (Kappmeyer et al., 1993; Knowles et al., 1991, 1992) belonging to major piroplasm surface protein (MPSP) family and which are conserved among the genus *Theileria* (Knowles et al., 1997). The EMA-1 and EMA-2 are glycosylphosphatidylinositol (GPI) anchored proteins with amino acid identity ranging from 17% to 55% in pairwise comparisons of family members (Kappmeyer et al., 2012). These EMAs have been utilized as diagnostic antigens in many ELISAs (Knowles et al., 1992; Baldani et al., 2011; Huang et al., 2003; Tanaka et al., 1999; Xuan et al., 2001).

The phylogenetic analysis on EMA-1 and 18S rRNA sequences has shown genetic diversity amongst different strains of *T. equi* circulating in various geographical regions and *T. equi* Mongolian strain genotype-4 has been identified as a new genetic clade based on EMA-1 sequences (Munkhjargal et al., 2013). The diagnostic assays developed from EMA-1/EMA-2 recombinant protein antigen, so far have largely utilized the USDA strain of *T. equi*. The genetic variation observed in EMA-1 gene of *T. equi* isolates from various geographies prompted us to examine one of the Asian strains of *T. equi* (Indian) for the expression of EMA-2 gene and develop a sensitive and specific serological assay for detection of *T. equi* antibodies. Such an ELISA developed employing Indian *T. equi* strain protein antigen would presumably be more sensitive to detect latently infected equines in endemic areas in different agro-climatic zones of the Indian states.

2. Materials and methods

2.1. The parasite DNA

Indian strain of *T. equi* (Kumar et al., 2008) has been maintained in a latently infected pony housed in large animal facility of the National Research Centre on Equines, Hisar. Red blood cells (RBCs) from this pony were used for DNA isolation by phenol–chloroform–isopropanol (PCI) method (Sambrook and Russell, 2001). Briefly, 200 μl of RBCs pellet was mixed with 10 volumes of DNA extraction buffer (0.1 M Tris-HCl, pH 8.0; 0.1 M NaCl; 10 mM EDTA containing 1% SDS). The contents of the tube were mixed by inverting it 10 times and then incubated at 55 °C for 2 h with proteinase K (10 mg/ml), followed by addition of equal volume of PCI. Finally, DNA from the aqueous phase was precipitated with ethanol and the pellet dried and dissolved in 50 μl of distilled water. The dissolved DNA sample was stored at −20 °C for further use in polymerase chain reaction (PCR).

2.2. Phylogenetic analysis

T. equi EMA-1 gene sequences were downloaded from NCBI GenBank database in FASTA format. These sequences were uploaded on MAFFT Online Server Program for multiple sequence alignment and further construction of phylogenetic tree using Neighbor-Joining method (Katoh et al., 2002). The confidence values of the branching patterns were estimated using bootstrap values, so as to evaluate genetic diversity.

2.3. PCR, cloning, expression and purification of T. equi (Indian strain) EMA-2 gene product

The nucleotide sequence of EMA-2 (JX024751, Indian strain) was used to design primers sequences. The PCR primers were designed so as to amplify a gene product of 693 bp (231AA) in PCR. The primer sequences included forward: 5′-gaattctgaggcaccaaaggtctc-3′ and reverse: 5′-gaattcaatccatcgtattgggtctt-3′. The underlined letters in the sequence of the primers represent the *Eco*RI linker site in the expression vector (pGEX 4T-1; Amersham Pharmacia Biotech, Piscataway, NJ). The PCR was performed at an annealing temperature of 58 °C for 45 s. The resulting gene product was digested with *Eco*RI, purified from the agarose gel (QIAquick gel extraction kit; Qiagen, Inc., Hilden, Germany), and further ligated into the *Eco*R1 site of pGEX-4T-1 expression vector. This resultant plasmid (pGEX/EMA-2-693) was further processed for the expression of recombinant gene products in *Escherichia coli* (DH5α strain), as a fused glutathione S-transferase (GST) protein, according to standard techniques (Sambrook and Russell, 2001). Briefly, the white *E. coli* colonies having recombinant plasmid pGEX/EMA-2-693 were picked and subjected to colony PCR (following the protocol described above). After verification, the positive colonies were further grown in Luria Bertani (LB) medium (1% bactotryptone, 0.5% yeast extract, 1% NaCl, and 0.1% (v/v) 5 N NaOH; supplemented with ampicillin sodium 50 μg/ml) at 37 °C till the OD_{600} reached between 0.3 and 0.5. Expression of the recombinant protein in *E. coli* was initiated by inducing the culture with 0.5 mM isopropyl-D-thiogalactopyranoside (IPTG) and incubating for a further 4 h at 37 °C. The GST tagged EMA-2 recombinant protein (TE/tEMA-2-GST) in *E. coli* was extracted by disrupting it with lysozyme (100 mg/ml) and 1% Triton X-100 contained in PBS (pH 7.0) along with sonication at 30 pulses for 3 min. The material was centrifuged at 10,000 × *g* for 15 min, and soluble supernatant fraction was collected. The recombinant protein in the above soluble fraction was allowed to bind with glutathione-Sepharose 4B (Wipro GE Healthcare Pvt Ltd, India) for 2 h at 4 °C. Finally, the recombinant EMA-2 protein was eluted from the Sepharose 4B beads by adding 10 mM glutathione solution. The supernatant was collected, and stored at −20 °C.

2.4. SDS-PAGE and Western Blot analysis

The purified EMA-2 recombinant protein (TE/tEMA-2-GST), *T. equi* infected horse's RBCs [*in vitro* (MASP technique) cultured *T. equi* infected horse RBCs], normal horse RBCs, *B. caballi* infected RBCs and GST protein were mixed separately with equal volume of 2× SDS sample buffer [62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 5% 2-mercaptoethanol, 2% SDS, and 0.01% bromophenol blue], and boiled for 5 min, followed by centrifugation at 8000 × *g* for 10 min. The supernatant was loaded on to SDS-PAGE (10% poly-acrylamide gel) and subjected to electrophoresis at a constant current of 60 mA for 60 min (Laemmli, 1970). The proteins in the gel were stained/destained

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or transferred (semi-dry electrophoretic transfer) to polyvinylidene fluoride (PVDF) membrane (Hybond-P, GE Healthcare Japan Corporation, Tokyo, Japan) at 0.8 mA/cm^2 as described previously (Towbin et al., 1979). 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 1 h 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.

2.5. EMA-2 enzyme linked immunosorbent assay ($_{EMA-2}$ELISA)

Each well of ELISA plate (Greiner Bio-one, Germany) was coated with 50 μl of recombinant protein (TE/tEMA-2GST) diluted in 1% BSA in PBS, pH 7.0 (1% BSA-PBS; at a final protein concentration of 2 μg/ml) and incubated overnight at 4 °C. The un-adsorbed antigenic sites were blocked by incubating the ELISA plate with 3% BSA in PBS followed by four washings with PBS containing 0.05% Tween 20 (PBS–T). The reference and test field serum samples were diluted to 1:200 in 1% BSA prepared in PBS, and added to the wells of the ELISA plate in duplicate, and incubated at room temperature (RT) for 1 h. After four washings with PBS–T, the ELISA plate was incubated with rabbit anti-horse IgG antibody conjugated with HRP diluted in 1% BSA-PBS at RT for 1 h. The ELISA plate was again washed four times with PBS–T and then 100 μl of substrate solution [3 mg O-phenylenediamine 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. Any sample showing an OD_{492} above the mean + 4*X* standard deviation (SD) of three negative wells was considered positive. The SD in the OD_{492} of three negative wells was calculated as per standard procedure (Snedecor and Cochran, 1989).

2.6. Validation

2.6.1. With cELISA

Serum samples were collected ($n = 120$) from different stud farms maintaining Indian Marwari or Thoroughbred breeds of horses. These samples were tested simultaneously with OIE recommended cELISA and $_{EMA-2}$ELISA developed in this study. The cELISA kit was imported from VMRD Inc., Pullman, WA, USA, and samples were analyzed following the manufacturer's instructions. The results obtained were compared for significance ($p < 0.05$) using Cohen's Kappa Coefficient (Snedecor and Cochran, 1989).

2.6.2. With Western blot

Western blot was performed to illustrate the specific antibody response against *T. equi* native antigen and EMA-2 recombinant protein. For this purpose, twenty five representative serum samples (out of 120 samples as above) were selected, which were detected positive ($n = 14$) or negative ($n = 11$) by both cELISA and $_{EMA-2}$ELISA. These serum samples were used in Western blot analysis. The EMA-2 recombinant antigen (TE/tEMA-2-GST), *T. equi* infected horse's RBCs (as above – native *T. equi* antigen) and normal horse RBCs were fractionated on SDS-PAGE and further processed for Western blot analysis (as described elsewhere) using these serum samples ($n = 25$).

2.7. Diagnostic sensitivity and specificity with respect to cELISA

The diagnostic sensitivity and specificity of the $_{EMA-2}$ELISA was evaluated against cELISA kit using 60 serum samples (Tyler and Cullor, 1989; Kumar et al., 2003). *T. equi* positive ($n = 33$) serum samples were selected from experimentally infected animals after appearance of *T. equi* parasites in blood smears. *T. equi* negative samples ($n = 27$) were selected from control animals which had been found negative for *T. equi* parasite by repeated blood smear examination and serologically by conventional ELISA (Kumar et al., 2013).

2.8. Reference serum samples

Two indigenous donkeys (1–1.5 years old) had been experimentally infected with *T. equi* and sequential serum samples were collected from day 0 to day 90 post-infection (PI). These serum samples were preserved and stored at −40 °C and were used in the present study for detecting antibodies against *T. equi* using $_{EMA-2}$ELISA. National Research Centre on Equines (NRCE), Hisar, India is maintaining three *T. equi* negative ponies in its animal housing facility [as confirmed by repeated blood smear examination and ELISA (using *T. equi* native antigen)] and their serum samples were used as reference negative control in $_{EMA-2}$ELISA. A reference *B. caballi* positive serum (1:80 complement fixation test, CFT titre) 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 and used in $_{EMA-2}$ELISA to test for cross-reactivity.

2.9. Field serum samples

A total of 5651 serum samples collected/received from indigenous equine population of 12 states of India (representing 8 Indian agro-climatic zones) during 2007–2012

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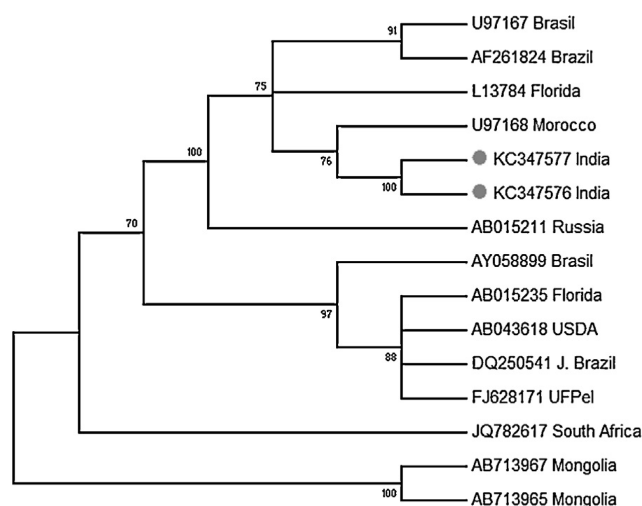

Fig. 1. Phylogenetic tree using *Theileria equi* EMA-1 gene sequences. This tree was constructed by Neighbor-Joining method with MAFFT program. Numbers shown at branch nodes indicate bootstrap values. This phylogenetic analysis showed high genetic diversity of EMA-1 gene among different strains of *T. equi*. A bullet has been placed before the EMA-1 sequences of Indian strain of *T. equi*.

were included to study the seroprevalence of *T. equi* antibodies using $_{EMA-2}$ELISA. Blood samples were transported on ice after collection; serum was separated in our laboratory by centrifuging at 3000 × *g* for 15 min and stored at 4 °C until further use. Prevalence was determined from the field data by analyzing 95% confidence interval (CI) and one-way ANOVA ($p < 0.05$) (Snedecor and Cochran, 1989).

3. Results

3.1. Phylogenetic analysis

T. equi EMA-1 nucleotide sequences from Indian strains were 100% similar to each other and 86–100% similar with Florida (L13784), Brazil (U97167), Morocco (U97168) strains and clustered together in phylogenetic tree (Fig. 1). Whereas, EMA-1 sequences from USDA (AB043618), Florida (AB015235), South Africa (JQ782617) and Mongolia (AB713967; AB713965) strains clustered separately (Fig. 1).

3.2. Recombinant protein expression and its purification (TE/tEMA-2-GST)

The PCR amplified product (693 bp) was sequenced and desired gene orientation was confirmed (data not shown). The recombinant plasmid clone expressed as 53 kDa GST fusion protein (TE/tEMA-2-GST) visualized on SDS-PAGE (Fig. 2A). The *T. equi* positive serum sample reacted with this recombinant expressed protein and *T. equi* infected horse RBCs only and not with normal and *B. caballi* infected horse RBCs or GST only protein (Fig. 2B).

3.3. $_{EMA-2}$ELISA and detection of specific antibodies in T. equi infected/non-infected serum samples

The optimal dilutions of equine serum samples and secondary HRP-conjugated anti-horse IgG were 1:200 and 1:10,000, respectively. The final OD_{492} of each sample clearly differentiated the *T. equi* antibody positive and negative equines (Fig. 2C). 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 $_{EMA-2}$ELISA and OD_{492} values were much the same as observed with *T. equi* reference negative serum samples (data not shown).

A significant rise in initial $_{EMA-2}$ELISA antibody end titre (1:400 onward) was observed from day 9 PI in the serum samples collected from both donkeys experimentally infected with *T. equi* parasites. The antibody titre in $_{EMA-2}$ELISA plateaued between 42 and 56 days PI (1:12,500) and remained at this level until the end of the experimental period of 90 days (Fig. 2D).

Table 1
Diagnostic sensitivity and specificity of cELISA and $_{EMA-2}$ELISA on serum samples of *Theileria equi* known disease status.

Parameters	cELISA	$_{EMA-2}$ELISA
Diagnostic sensitivity[a] (DSn)	0.95 (31/33)	0.97 (32/33)
Diagnostic specificity[b] (DSp)	0.96 (26/27)	0.96 (26/27)

Note: figures in parenthesis indicate number of serum samples reacted out of total samples tested.
[a] Probability of accurately identifying true-positive (diseased) animals out of 33 *T. equi* disease-positive samples.
[b] Probability of accurately identifying true-negative (normal) animals out of 27 *T. equi* disease-negative samples.

3.4. Validation

The results as observed in $_{EMA-2}$ELISA did not differ significantly ($p < 0.05$) from cELISA on corresponding 120 serum samples. A very high degree of kappa coefficient (‘κ’ = 0.93) along with positive linear relationship between corresponding samples $_{EMA-2}$ELISA OD_{492} values and cELISA per cent inhibition (Fig. 3A) were obtained indicating absolute validation of $_{EMA-2}$ELISA assay developed in this study. The results of the $_{EMA-2}$ELISA, cELISA and Western blot analysis were in complete agreement on all the 25 serum samples tested. A potent antibody response was detected against the recombinant protein and *T. equi*-specific polypeptides, whereas no antibody response was observed against normal horse erythrocytic stroma in immunoblot analysis (Fig. 3B), which implied that the expressed recombinant protein has high specificity for *T. equi*-specific antibodies only. The *T. equi* positive serum samples reacted strongly with *T. equi* specific polypeptides – 18, 30, 34 and 56 kDa (Fig. 3).

3.5. Diagnostic sensitivity, specificity and cross-reactivity

The diagnostic sensitivity of cELISA and $_{EMA-2}$ELISA was 0.95 and 0.97, respectively, while diagnostic specificity (0.96) was the same for both the assays (Table 1). There were a few samples which were *T. equi* disease positive but detected negative in cELISA (two samples) and $_{EMA-2}$ELISA (one sample). These samples represented early phase of *T. equi* infection in experimentally infected donkeys (collected during 12–18 days PI). Similarly, one sample tested

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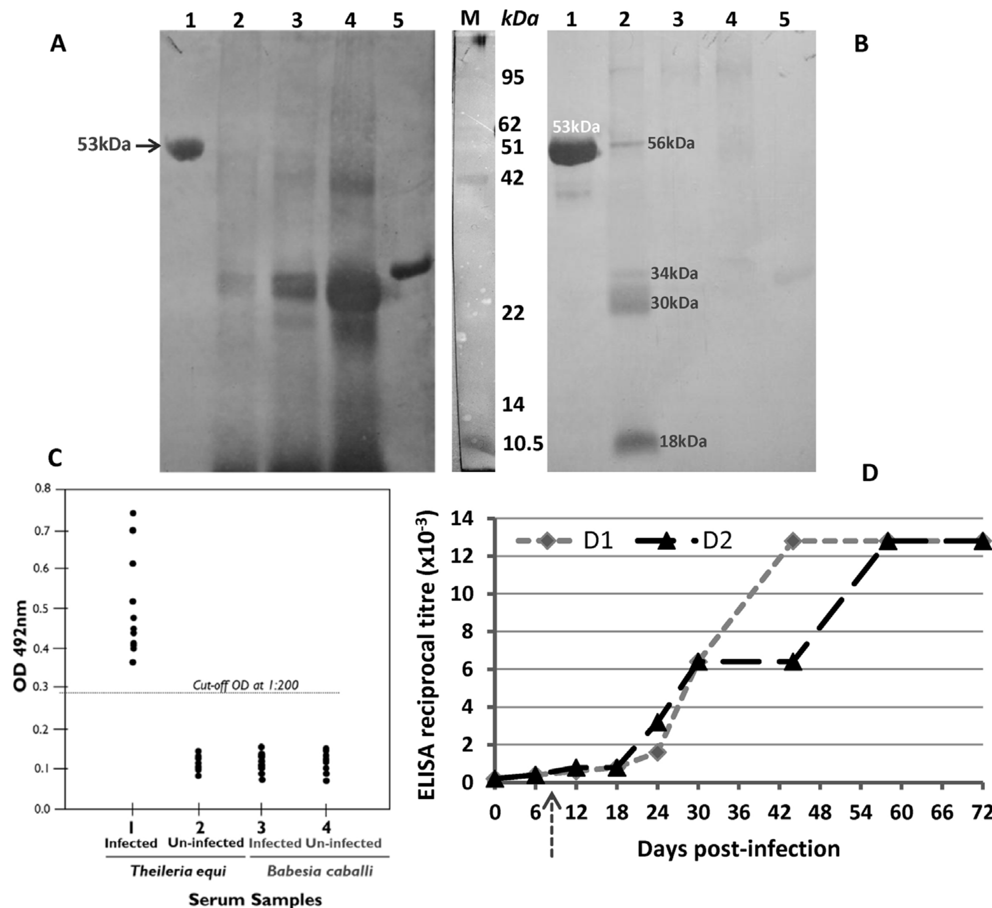

Fig. 2. Expression and purification of EMA-2 recombinant protein (TE/tEMA-2-GST) and its antigenic reactivity on Western blot analysis and in $_{EMA-2}$ELISA. (A) Antigens on SDS-PAGE and stained with Amido Black 10B, TE/tEMA-2-GST recombinant protein was of expected size, *i.e.* 53 kDa [~27 kDa-truncated EMA-2 protein and 26 kDa-GST tag]. (B) Reactivity of TE/tEMA-2-GST and *T. equi* infected RBCs to serum from *T. equi*-infected horse by Western blot analysis. Lanes 1: TE/tEMA-2-GST, 53 kDa protein; 2: *T. equi* infected horse RBCs; 3: Normal horse RBCs; 4: *Babesia caballi* infected horse RBCs; 5: GST protein; Lane M: Pre-stained molecular weight marker (10.5–175 kDa). (C) The TE/tEMA-2-GST antigen immune-reacted with *T. equi* positive serum in ELISA and not with *B. caballi* positive serum. (D) Sequential serum samples from *T. equi* infected donkeys detected specific antibodies as early as day 9 PI and highest antibody titre observed was 1:12,500. Antibody titres were expressed as the highest serum dilutions which showed an OD_{492} of >0.3.

positive both in cELISA and $_{EMA-2}$ELISA, while it was of *T. equi* negative disease status.

3.6. Seroprevalence of *T. equi* antibody among Indian equids

We conducted seroprevalence studies on equine serum samples collected from 12 states of India *viz.*, Jammu & Kashmir, Himachal Pradesh, Punjab, Haryana, Rajasthan, Gujarat, Uttarakhand, Uttar Pradesh, Madhya Pradesh, Chhattisgarh, Karnataka and Sikkim. A total of 5651 serum samples – collected during the years 2007–2012 – were included for assessing seroprevalence. Out of 5651 equine serum samples tested by $_{EMA-2}$ELISA, 1845 (32.65%) were found positive for *T. equi* antibodies (Table 2). The per cent seroprevalence differed significantly year-to-year, as the positivity was affected by geographical area/endemic zone of the collected sample (Table 3).

4. Discussion

Different forms of ELISAs have been described based on *T. equi* native antigen or recombinant proteins. The success of specific antibody detection in ELISA depends on absolute early detection of latent infection. In a previous study it has been demonstrated that EMA-1 and EMA-2 mutually expressed on the surface of extra-/intra-erythrocytic merozoites, and that the intra-erythrocytic merozoites shed only EMA-2 antigen in the infected erythrocytic cytoplasm or inside the membrane surface (Kumar et al., 2004). Recent experiments confirmed co-precipitation of horse erythrocytic actin molecule with recombinant EMA-2 protein, but not with the EMA-1 protein (Kumar et al., 2012). These novel developments prompted us to develop an ELISA based on recombinant protein derived from EMA-2 merozoite surface protein of *T. equi*. We sequenced EMA-2 gene from an Indian strain of *T. equi* (AFN08856), which is 94% and 97% identical with EMA-2 protein sequence of USDA (O61162) and Florida (O44071, Q9TY24) strains of *T. equi*, respectively (Altschul et al., 1997; DDBJ BLASTP analysis). Genetic diversity based on EMA-1 and 18s rRNA gene of *T. equi* has been reported from South Africa (Bhoora et al., 2010) and Mongolia (Munkhjargal et al., 2013). The EMA-1 sequences (KC347576, KC347577) from Indian strain of *T. equi* also showed genetic diversity when compared with other strains of *T. equi* (Fig. 1). In the GenBank database,

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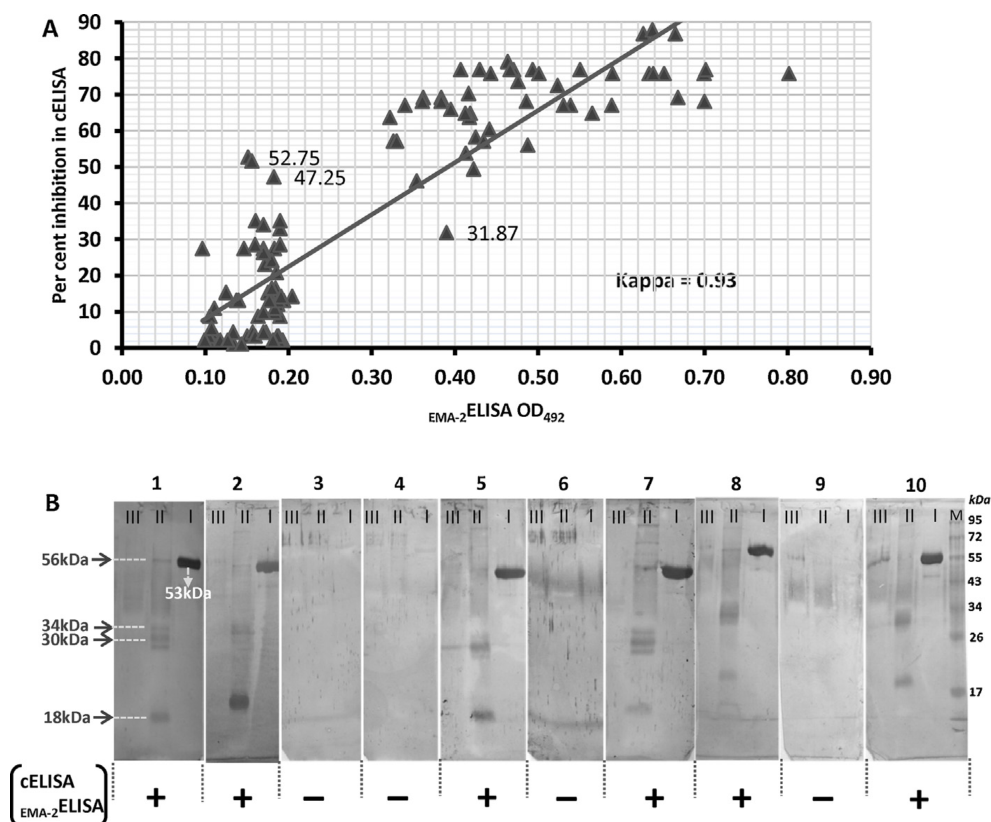

Fig. 3. Validation of $_{EMA-2}$ELISA OD_{492} with corresponding per cent inhibition in cELISA and Western blot analysis on representative serum samples. (A) Scattered graph with linear line between $_{EMA-2}$ELISA OD_{492} and per cent inhibition in cELISA on corresponding 120 serum samples. The corresponding sample spot showed very high degree of kappa coefficient (0.93) indicating absolute correlation. (B) TE/tEMA-2-GST recombinant protein (lane I), *T. equi* infected horse RBCs (lane II) and non-infected horse RBCs (lane III) were immuno-blotted individually with ten representative serum samples and corresponding $_{EMA-2}$ELISA and cELISA are also shown.

only a few EMA-2 sequences of *T. equi* are available for phylogeny analysis to assess genetic diversity. Accordingly, PCR primers were designed from EMA-2 (AFN08856) sequence and gene product was expressed and recombinant protein was used in development of immunoassay for sero-epidemiological studies.

The bacterial expression system was used for expression of truncated EMA-2 gene of *T. equi* gene, as it is most preferred system for laboratory investigation and promises large amount of purified expressed protein (Chen, 2012). A 53 kDa GST-tagged EMA-2 recombinant protein was obtained (TE/tEMA-2-GST), reacting distinctly with anti-*T.*

Table 2
Seroprevalence of *Theileria equi* antibodies as detected by $_{EMA-2}$ELISA in equine serum samples collected during 2007–2012 according to agro-climatic zones of India.

	Indian Agro-climatic zones	States in India	Samples tested (positive/total samples tested)	Per cent prevalence	CI[a]
1	Western Himalayan Region	Jammu & Kashmir Himachal Pradesh	130/794 31/109	17.82	15.32–20.32
2	Middle Gangetic plains Region	Uttar Pradesh Chandigarh	111/378 10/39	29.02	24.66–33.38
3	Upper Gangetic Plains Region	Uttarakhand	141/582	24.23	20.75–27.71
4	Trans Gangetic plains Region	Haryana Punjab Chandigarh	159/348 44/119 10/39	42.09	37.79–46.39
5	Central Plateau & Hills Region	Madhya Pradesh	198/552	35.87	31.87–39.87
6	West coast plains & Hills Region	Maharashtra	46/328	14.02	10.26–17.78
7	Gujarat Plains & Hills Region	Gujarat	220/438	50.23	45.55–54.91
8	Western Dry Region	Rajasthan	701/1819	38.54	36.30–40.78
	Total		1845/5651	32.65	31.43–33.87

[a] 95% confidence interval (CI) range for true population proportion.

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Table 3
Annual seroprevalence of *Theileria equi* antibodies as detected by $_{EMA\text{-}2}$ELISA in equine serum samples collected during 2007–2012.

Year	Samples tested (positive/total samples tested)	Per cent prevalence	CI[a]
2007	170/698	24.36[a]	21.18–27.54
2008	202/781	25.86[a]	22.79–28.93
2009	320/840	38.10[b]	34.82–41.38
2010	331/908	36.45[b]	33.32–39.58
2011	421/1146	36.74[b]	33.95–39.53
2012	401/1278	31.37[c]	28.83–33.91
Total	1845/5651	32.64	31.43–33.87

The figures having different superscripts letters differ significantly ($p < 0.05$).
[a] 95% confidence interval (CI) range for true population proportion.

equi horse serum and not with anti-*B. caballi* horse serum. Serum sample collected from *T. equi* latently infected horse reacted potentially with *T. equi* infected RBCs only and not with normal/*B. caballi* infected RBCs or GST antigen, indicating high specificity of the expressed recombinant protein (TE/tEMA-2-GST). The $_{EMA\text{-}2}$ELISA was developed using TE/tEMA-2-GST recombinant protein and it clearly distinguished *T. equi* reference positive and negative serum samples. Usually the negative samples OD_{492} values were <0.2, whereas positive samples OD_{492} were >0.35. No cross-reaction was observed with reference positive serum for *B. caballi* or *T. evansi* or other equine bacterial/viral pathogens. The $_{EMA\text{-}2}$ELISA detected *T. equi*-specific antibodies in experimentally infected donkeys from days 9 to 12 PI onward (antibody titre >1:400 considered positive), whereas, parasite was observed a bit earlier, *i.e.* 3–6 days PI (Kumar et al., 2013). Similar findings in the ELISA, using *T. equi* recombinant or native antigen have also been reported by Hirata et al. (2002), Huang et al. (2003) and Kumar et al. (1997, 2003).

The OIE (2008) has prescribed cELISA as an official assay for detecting antibodies against *T. equi* in horses intended for international trade. However, the immunoblot analysis has been found to be more specific than cELISA and CFT, when testing response to treatment against *B. caballi* in horses (Schwint et al., 2009). Schwint et al. (2009) treated four horses for *B. caballi* infection and in two horses the immunoblot analysis detected specific antibody for longer period than was observed with cELISA. Accordingly, we preferred cELISA and Western blot analysis for validation of the results obtained in $_{EMA\text{-}2}$ELISA. Almost perfect agreement (kappa = 0.93) was obtained between $_{EMA\text{-}2}$ELISA OD_{492} and per cent inhibition in cELISA, indicating the suitability of $_{EMA\text{-}2}$ELISA developed in this study for serodiagnosis and seroprevalence studies. Results of four serum samples did not proportionate with corresponding observations in $_{EMA\text{-}2}$ELISA and cELISA, however rest of the sample's results (96.6%) were in agreement between these two assays. Similar findings were also reported by Farkas et al. (2013) and Shkap et al. (1998) when comparing results of cELISA with IFAT. The high diagnostic sensitivity and specificity implied suitability of these assays in explicit identification of *T. equi* infected animals (true-positive) and *T. equi* non-infected animals (true-negative), respectively. A few serum samples had low OD_{492} or per cent inhibition values indicating the presence of low circulating *T. equi*-specific antibodies (Kumar et al., 2003), and demonstration of *T. equi* parasite by macro-aerophilous stationary phase culture (MASP) technique in such samples may expand the confidence in sensitivity of these assays.

In seroprevalence studies on equines serum samples collected from 8 agro-climatic zones and 12 states of India, a high prevalence of *T. equi* antibodies (32.65%) were detected by $_{EMA\text{-}2}$ELISA. Higher seroprevalence of *T. equi* antibody was observed in equines from the Gujarat plains and Hills region (50.33%); Trans-Gangetic plains (42.09%); Western dry region (38.54%), and Central plateau and hills region (35.87%). Previously, Malhotra et al. (1978) also recorded high prevalence of *T. equi* in Rajasthan followed by Uttar Pradesh and Haryana and it is noteworthy that these states fall in these agro-climatic zones of India. Furthermore, *Hyalomma anatolicum anatolicum* is the principal vector tick of *T. equi* in horses (Bhattacharylu et al., 1975; Chaudhuri et al., 1969; Kumar et al., 2007) and climatic conditions of these agro-climatic zones are well suited for of the maintenance of this tick species (Geeverghese et al., 1997).

It can be concluded from this study that TE/tEMA-2-GST recombinant protein-based $_{EMA\text{-}2}$ELISA is sensitive and specific in detecting *T. equi* antibodies in equine serum samples. This assay has been applied on serum samples collected from different agro-climatic zones of India and have demonstrated that a sizeable Indian equine population is carrying *T. equi* specific antibodies. Hence, this $_{EMA\text{-}2}$ELISA assay is suitable for use in the laboratory for sero-diagnosis of *T. equi* infection in equine samples and has promising potential for wide application in other Asian countries.

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References

Altschul, S.F., Thomas, L.M., Alejandro, A.S., Jinghui, Z., Zheng, Z., Webb, M., David, J.L., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.
Baldani, C.D., Hilario, E., Nakaghi, A.C., Bertolini, M.C., Machado, R.Z., 2011. Production of recombinant EMA-1 protein and its application for the

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diagnosis of *Theileria equi* using an enzyme immunoassay in horses from São Paulo State, Brazil. Rev. Bras. Parasitol. Vet. 20, 54–60.
Bhattacharylu, Y., Chaudhuri, R.P., Gill, B.S., 1975. Transtadial transmission of *Theileria annulata* through common ixodid ticks infesting Indian cattle. Parasitology 71, 1–7.
Bhoora, R., Quan, M., Matjila, P.T., Zweygarth, E., Guthrie, A.J., Collins, N.E., 2010. Sequence heterogeneity in the equi merozoite antigen gene (ema-1) of *Theileria equi* and development of an ema-1-specific TaqMan MGB assay for the detection of *T. equi*. Vet. Parasitol. 172, 33–45.
Chahan, B., Zhang, S., Seo, J.Y., Nakamura, C., Zhang, G., Bannai, H., Jian, Z., Inokuma, H., Tuchiya, K., Sato, Y., Kabeya, H., Maruyama, S., Mikami, T., Xuan, X., 2006. Seroepidemiological evidence for the possible presence of *Babesia* (*Theileria*) *equi* and *Babesia caballi* infections in donkeys in western Xinjiang, China. J. Vet. Med. Sci. 68, 753–755.
Chaudhuri, R.P., Srivastava, S.C., Naithani, R.C., 1969. On the biology of the ixodid tick Hyalomma (Hyalomma) anatolicum anatolicum Koch, 1844 (Acarina: Ixodidae). Indian J. Anim. Sci. 39, 257–268.
Chen, R., 2012. Bacterial expression systems for recombinant protein production: *E. coli* and beyond. Biotechnol. Adv. 30, 1102–1107.
De Waal, D.T., 1992. Equine piroplasmosis: a review. Br. Vet. J. 148, 6–14.
Farkas, R., Tánczos, B., Gyurkovszky, M., Földvári, G., Solymosi, N., Edelhofer, R., Hornok, S., 2013. Serological and molecular detection of *Theileria equi* infection in horses in Hungary. Vet. Parasitol. 192, 143–148.
Friedhoff, K.T., Tenter, A.M., Muller, I., 1990. Haemoparasites of equines: impact on international trade of horses. Rev. Sci. Tech. – Off. Int. Epizoot. 9, 1187–1194.
Gautam, O.P., Dwivedi, S.K., 1976. Equine babesiosis: a severe outbreak in stud farm at Hisar. Indian Vet. J. 53, 546–551.
Geeverghese, G., Fernandez, S., Kulkarni, S.M., 1997. A checklist of Indian ticks. Indian J. Anim. Sci. 67, 566–574.
Hailat, N.Q., Lafi, S.Q., al-Darraji, A.M., al-Ani, F.K., 1997. Equine babesiosis associated with strenuous exercise: clinical and pathological studies in Jordan. Vet. Parasitol. 69, 1–8.
Hirata, H., Ikadai, H., Yokoyama, N., Xuan, X., Fujisaki, K., Suzuki, N., Mikami, T., Igarashi, I., 2002. Cloning of a truncated *Babesia equi* gene encoding an 82-kilodalton protein and its potential use in an enzyme-linked immunosorbent assay. J. Clin. Microbiol. 40, 1470–1474.
Huang, X., Xuan, X., Yokoyama, N., Xu, L., Suzuki, H., Sugimoto, C., Nagasawa, H., Fujisaki, K., Igarashi, I., 2003. High-level expression and purification of a truncated merozoite antigen-2 of *Babesia equi* in *Escherichia coli* and its potential in immunodiagnosis. J. Clin. Microbiol. 41, 1147–1151.
Kappmeyer, L.S., Perryman, L.E., Knowles Jr., D.P., 1993. A *Babesia equi* gene encodes a surface protein with homology to *Theileria* species. Mol. Biochem. Parasitol. 62, 121–124.
Kappmeyer, L.S., Thiagarajan, M., Herndon, D.R., Ramsay, J.D., Caler, E., Djikeng, A., Gillespie, J.J., Lau, A.O., Roalson, E.H., Silva, J.C., Silva, M.G., Suarez, C.E., Ueti, M.W., Nene, V.M., Mealey, R.H., Knowles, D.P., Brayton, K.A., 2012. Comparative genomic analysis and phylogenetic position of *Theileria equi*. BMC Genomics 13, 603–616.
Katoh, K., Misawa, K., Kuma, K., Miyata, T., 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 30, 3059–3066.
Knowles, D.P., 1996. Equine Babesiosis (Piroplasmosis): a problem in the international movement of horses. Br. Vet. J. 152, 123–126.
Knowles Jr., D.P., Kappmeyer, L.S., Stiller, D., Hennager, S.G., Perryman, L.E., 1992. Antibody to a recombinant merozoite protein epitope identified horses infected with *Babesia equi*. J. Clin. Microbiol. 30, 3122–3126.
Knowles, D.P., Kappmeyer, L.S., Perryman, L.E., 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.
Knowles Jr., D.P., Perryman, L.E., Kappmeyer, L.S., Hennager, S.G., 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, 2056–2058.
Kumar, S., Kumar, R., 2007. Diagnosis of *Babesia equi* infection in equines: an update on the methods available. CAB Rev.: Perspect. Agric. Vet. Sci. Nutr. Nat. Resour. 2, 1–14.
Kumar, S., Malhotra, D.V., Dhar, S., 1997. Serodiagnosis of *Babesia equi* infection – a comparison of Dot-ELISA, complement fixation test and capillary tube agglutination test. Vet. Parasitol. 69, 171–176.
Kumar, S., Kumar, Y., Malhotra, D.V., Dhar, S., Nichani, A.K., 2003. Standardization and comparison of serial dilution and single dilution enzyme linked immunosorbent assay (ELISA) using different antigenic preparations of the *Babesia* (*Theileria*) *equi* parasite. Vet. Res. 34, 71–83.
Kumar, Y., Malhotra, D.V., Nichani, A.K., Dhar, S., Kumar, S., 2013. Immunokinetics of *Theileria equi* specific antibodies – a comparison in serial and single dilution ELISA antibody end titres. Turk. J. Vet. Anim. Sci. 37, 429–433.
Kumar, S., Malhotra, D.V., Sangwan, A.K., Goel, P., Kumar, A., Kumar, S., 2007. Infectivity rate and transmission potential of *Hyalomma anatolicum anatolicum* ticks for *Babesia equi* infection. Vet. Parasitol. 144, 338–343.
Kumar, S., Kumar, R., Gupta, A.K., Dwivedi, S.K., 2008. Passive transfer of *Theileria equi* antibodies to neonate foals of immune tolerant mares. Vet. Parasitol. 151, 80–85.
Kumar, S., Yokoyama, N., Kim, J.Y., Bork-Mimm, S., Inoue, N., Xuan, X., Igarashi, I., Sugimoto, C., 2012. *Theileria equi* merozoite antigen-2 interacts with actin molecule of equine erythrocyte during their asexual development. Exp. Parasitol. 132, 508–512.
Kumar, S., Yokoyama, N., Kim, J.Y., Huang, X., Inoue, N., Xuan, X., Igarashi, I., Sugimoto, C., 2004. Expression of *Babesia equi* EMA-1 and EMA-2 during merozoite developmental stages in erythrocyte and their interaction with erythrocytic membrane skeleton. Mol. Biochem. Parasitol. 133, 221–227.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nat. (Lond.) 227, 680–685.
Malhotra, D.V., Gautam, O.P., Banerjee, D.P., 1978. Prevalence of latent cases of *Babesia equi* infection in some parts of North-West India as measured by capillary tube agglutination test. Equine Vet. J. 10, 24–26.
Munkhjargal, T., Sivakumar, T., Battsetseg, B., Nyamjargal, T., Aboulaila, M., Purevtseren, B., Bayarsaikhan, D., Byambaa, B., Terkawi, M.A., Yokoyama, N., Igarashi, I., 2013. Prevalence and genetic diversity of equine piroplasms in Tov province, Mongolia. Infect. Genet. Evol. 16C, 178–185.
Office International des Epizooties (OIE), 2008. Equine piroplasmosis. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 5th ed. Office International des Epizooties, Paris, France, pp. 884–893.
Rashid, A., Mubarak, A., Hussain, A., 2009. Babesiosis in equines in Pakistan: a clinical report. Vet. Ital. 45, 391–395.
Sambrook, J., Russell, D.W., 2001. Molecular Cloning a Laboratory Manual, 3rd ed. Cold Spring Harbour Laboratory Press, New York.
Schwint, O.N., Uiti, M.W., Palmer, G.H., Kappmeyer, L.S., Hines, Mt., Cordes, R.T., Knowles, D.P., Scoles, G.A., 2009. Imidocarb dipropionate clears persistent *Babesia caballi* infection with elimination of transmission potential. Antimicrob. Agents Chemother. 53, 4327–4332.
Sharma, R.D., Gautam, O.P., 1977. Clinical cases of equine babesiosis. Haryana Vet. 16, 19–26.
Sharma, R.D., Tanwar, R.K., Gahlot, A.K., Yadav, J.S., Rajvanshi, D.S., 1982. Note on clinical studies in *Babesia equi* infection in ponies. Indian J. Anim. Sci. 52, 1001–1003.
Shkap, V., Cohen, I., Leibovitz, B.S., Pipano, E., Avni, G., Shofer, S., Giger, U., Kappmeyer, L., Knowles, D.P., 1998. Seroprevalence of *Babesia equi* among horses in Israel using competitive inhibition ELISA and IFA assays. Vet. Parasitol. 76, 251–259.
Snedecor, G.W., Cochran, W.G., 1989. Statistical Methods. Affiliated East-West Press Pvt. Ltd, New Delhi.
Tanaka, T., Xuan, X., Ikadai, H., Igarashi, I., Nagasawa, H., Fujisaki, K., Mikami, T., Suzuki, N., 1999. Expression of *Babesia equi* merozoite antigen-2 by recombinant baculovirus and its use in the ELISA. Int. J. Parasitol. 29, 1803–1808.
Towbin, H., Stachelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. 76, 4350–4354.
Tyler, J.M., Cullor, J.S., 1989. Titers, tests, and truisms: rational interpretation of diagnostic serological testing. J. Am. Vet. Med. Assoc. 194, 1550–1558.
Ueti, M.W., Mealey, R.H., Kappmeyer, L.S., White, S.N., Kumpula-McWhirter, N., Pelzel, A.M., Grause, J.F., Bunn, T.O., Schwartz, A., Traub-Dargatz, J.L., Hendrickson, A., Espy, B., Guthrie, A.J., Fowler, W.K., Knowles, D.P., 2012. Re-emergence of the apicomplexan *Theileria equi* in the United States: elimination of persistent infection and transmission risk. PLoS ONE 7, e44713.
Xu, Y., Zhang, S., Huang, X., Bayin, C., Xuan, X., Igarashi, I., Fujisaki, K., Kabeya, H., Maruyama, S., Mikami, T., 2003. Seroepidemiologic studies on *Babesia equi* and *Babesia caballi* infections in horses in Jilin province of China. J. Vet. Med. Sci. 65, 1015–1017.
Xuan, X., Larsen, A., Ikadai, H., Tanaka, T., Igarashi, I., Nagasawa, H., Fujisaki, K., Toyoda, Y., Suzuki, N., Mikami, T., 2001. Expression of *Babesia equi* merozoite antigen 1 in insect cells by recombinant baculovirus and evaluation of its diagnostic potential in an enzyme-linked immunosorbent assay. J. Clin. Microbiol. 39, 705–709.