

Evaluation of pre-erythrocytic stage recombinant proteins of *Theileria annulata* for early diagnosis of bovine tropical theileriosis in Indian cattle

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ABSTRACT

A study was carried out to evaluate 2 pre-erythrocytic recombinant proteins for the diagnosis of bovine tropical theileriosis during the initial phase of the disease. *Theileria annulata* surface protein (*TaSP*) and the C terminal region of SPAG–1 were cloned and expressed as fusion with thioredoxine in *E. coli*. The proteins were purified to near homogeneity using Ni-NTA agarose affinity chromatography. In ELISA, recombinant *TaSP* (*rTaSP*) was able to detect antibodies from 7 to 14 days onward in all the 4 experimental bovine calves while no significant results were obtained with rSPAG–1 (C-terminus). *rTaSP* also showed higher sensitivity than crude piroplasmic antigen in 50 field sera by ELISA. In addition, *rTaSP* did not show any cross reactivity with *Babesia bigemina* and *Trypanosoma evansi* positive sera. The present findings indicated *rTaSP* as a potential molecule for serodiagnosis of *T. annulata*.

Key words: Bovine tropical theileriosis, ELISA, India, Recombinant, Serodiagnosis, SPAG–1, *TaSP*, *Theileria annulata*

Tropical bovine theileriosis, caused by *Theileria annulata*, an intracellular protozoan parasite is one of the most economically important livestock diseases in India, costing the country as much as US \$ 239.5 million annually (Minjauw and McLeod 2003). The disease is characterized by high temperature, swollen lymph nodes, splenomegaly and progressive anaemia. Crossbred cattle are considered more susceptible to theileriosis than the indigenous breed and the disease has been reported in cattle from almost all parts of the country (Chandra *et al.* 2000, Bansal 2005). Presently, in India, diagnosis of theileriosis is primarily made either by blood smear examination or serological assays based on crude parasite extracts. To overcome the limitations of serodiagnosis using crude extract, the recombinant proteins derived from *T. annulata* were used in an attempt to develop reliable diagnostic test.

MATERIALS AND METHODS

Parasite stock and RNA isolation: For isolation of RNA, macroschizont infected with Parbhani strain of *T. annulata*

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was used. The infected lymphoblastoid cells were cultured in RPMI 1640 media supplemented with 10% inactivated bovine serum and antibiotics (penicillin @ 100 U/ml and streptomycin @ 100 µg/ml of medium) at 37°C. RNA was isolated using Tri Reagent from approximately 5×10⁶ cells according to the manufacturer's instruction. The purity and concentration were checked spectrophotometrically.

Reverse transcription polymerase chain reaction amplification of *TaSP* gene: The entire ORF of *TaSP* gene was reverse transcribed from approximately 1µg of total RNA using Primer SPF_R1 and subsequently the cDNA was PCR amplified with primer SPF_F1 following the protocol of Sambrook and Russell (2001). The amplified PCR product was ligated into a cloning vector and transformed into DH5α *E. coli* cells. Positive clones were sequenced and stored for further use. The sequence of *TaSP* cDNA used in the present study has been submitted to Genbank (Accession No. EF070732).

***E. coli* expression of recombinant *TaSP* and SPAG–1:** Both the recombinant proteins were expressed fused with thioredoxine and histidine tags using pET32c (+) expression vector. Truncated *TaSP* (Schnittger *et al.* 2002) was amplified from the cDNA clone using the primer pair of pTSP_F and SPH_R2. The amplified fragment was cloned into pGEMT Easy cloning vector and subsequently released by *Nco*I and *Sac*I restriction digestion. The fragment was ligated into a

similarly digested expression vector pET32c (+) and transformed into a BL-21 pLys S *E. coli* cells.

The C-terminal fragment of SPAG-1 gene (Williamson *et al.* 1989) was amplified from the cloned SPAG-1 cDNA already available in the laboratory. The gene fragment was amplified using primer SR1_F and SR1_R, digested with *Bam*HI and *Hind*III restriction enzymes, and cloned into pET32c (+) expression vector. Expressions of both the recombinant proteins were induced in *E. coli* with 1 mM IPTG. The recombinant proteins were purified with affinity chromatography using Ni-NTA agarose. Purity of the recombinant proteins was verified on SDS-PAGE. The reactivity of the recombinant proteins was evaluated by western blotting. Recombinant *Ta*SP (r*Ta*SP) was allowed to react with infected cattle serum and recombinant SPAG-1 (rSPAG-1) with hyper immune serum against ground up tick tissue sporozoites (GUTTS) available in laboratory.

All the PCR reactions were carried out at 95°C for 5 min for initial denaturation followed by 30 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min.

Piroplasm antigen: Crude extract of piroplasm was prepared as described by Ray *et al.* (1998) and stored at 4°C till further use.

Serum samples: Serum samples were collected from 4 crossbred bovine calves, aged 6 to 8 months, experimentally infected with 2 tick equivalent of GUTTS of *T. annulata* (Parbhani strain). The animals were housed in a tick proof shed and monitored for clinical signs. Microscopical examination of lymph node biopsy and thin blood smear was carried out regularly. Serum was collected in weekly intervals for 4 weeks and stored at -20°C until further use. Fifty bovine field sera samples were collected from endemic region for screening by ELISA with r*Ta*SP keeping crude piroplasm antigen as the reference antigen. Serum samples, 3 each, from bovine calves experimentally infected with either *Babesia bigemina* or *Trypanosoma evansi* were also collected for testing cross reactivity of r*Ta*SP in ELISA.

Enzyme-linked immunosorbent assay: Approximately, 50 µg of the recombinant antigens and 500 ng of piroplasm antigen were used to coat wells of 96 micro ELISA plate.

Table 1. Sequences of oligonucleotide primers used

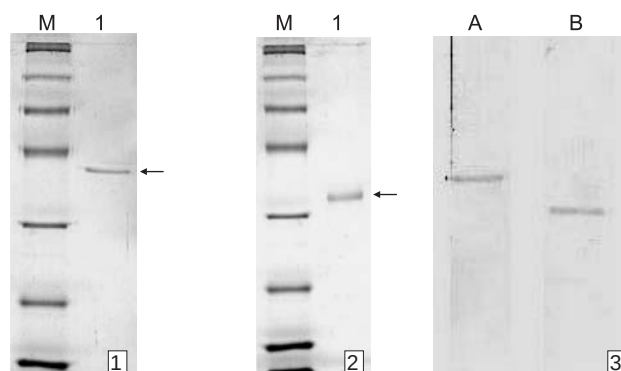
SPF_F1	5'-GGACGGATCCATGAAATTCTTCTACCTTTT GTT -3'
SPF_R1	5'- CGCTGCAGTTAACAACAATCTTCGTTAATGC-3'
pTSP_F	5'-GTCGACCATGGATCGACAACCTTAATCC-3'
SPH_R2	5'-ATCTGCAGTACCCGTCAGACTCATCATC-3'
SR1_F	5'-AACTGCAGGATCCATGAACTAGTGAAGGA-3'
SR1_R	5'-GAATTCAAGCTTTTAGTGGACTATGCTG-3'

The plate was incubated at 4°C overnight and the excess antigen was removed by washing 3 times with PBS (pH 7.2) containing Tween20 (PBST). Blocking of the non-reacting sites was done with 3% skimmed milk, prepared in PBST, for 1 h at 37°C. After washing the plates, serum samples diluted 1 : 100 with PBST and 1% skimmed milk were loaded into each well and incubated for 1 h at 37°C. The unbound antibodies were washed out times with PBST. Antibovine IgG horseradish peroxidase conjugate diluted 1 : 5000 with PBST and 1% skimmed milk was added to each well and incubated further for 1 h at 37°C. After giving a final wash, 100 µl of OPD substrate in citrate phosphate buffer with H₂O₂ was added to each well. The reaction was allowed for 15 min after which 50 µl of 3 N HCl was added to stop the reaction. Absorbance was recorded at 490 nm using ELISA reader.

Preinfection sera from the 4 experimental bovine calves were used as a negative control for study on experimental calves whereas in addition to the 4 negative samples, 10 negative sera from infection-free bovine calves were used as negative control for the field samples. All the samples were loaded in duplicate wells. The average reading of the negative control plus two standard deviations was considered a cut off value for the tests.

RESULTS AND DISCUSSION

A sensitive and specific diagnostic system is essential for effective control of any disease. The recombinant proteins evaluated here in natural state, are expressed in the early stages of the parasite life cycle; hence, they are expected to recognise infection specific antibodies earlier than those proteins which are expressed in the later stages. These proteins were cloned and expressed in *E. coli*. SDS-PAGE analysis revealed the approximate size of the fused recombinant proteins as 40 and 33 kDa for *Ta*SP and C-terminus of SPAG-1 respectively (Figs 1, 2). Both the



Figs 1-3. 1. SDS-PAGE analysis of purified r*Ta*SP; Lane M: Molecular weight marker; Lane 1: Purified r*Ta*SP; 2. SDS-PAGE analysis of purified rSPAG-1 (C-terminus); Lane M: Molecular weight marker; Lane 1: Purified rSPAG-1 (C-terminus); 3. Western blot reaction of recombinant proteins. Lane 1: r*Ta*SP, Lane 2: rSPAG-1 (C-terminus).

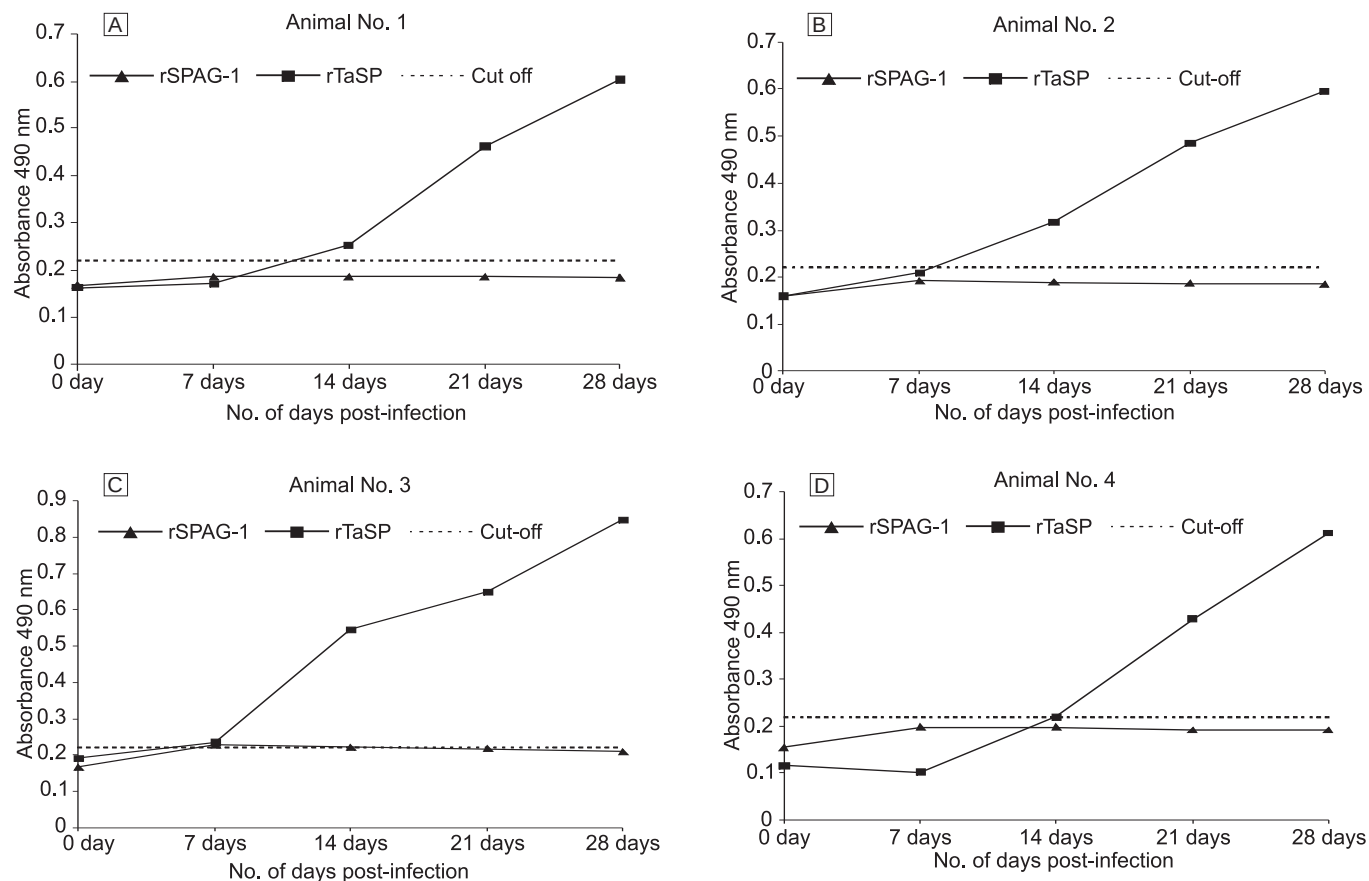


Fig. 4 (A to D): ELISA O.D. pattern of the 4 experimental calves against the recombinant proteins.

recombinant proteins reacted with the respective serum in western blotting (Fig. 3).

All the experimental calves developed clinical theileriosis and manifested symptoms of fever and swelling of lymph nodes. Erythrocytic parasitaemia was observed on blood smear examination from all the animals. *TaSP* was able to detect antibodies between day 7 and 14 of infection in all the 4 experimental calves. The O.D. reading of the samples maintained an upward trend for the rest of the experiment in all the animals. On the other hand, we could not establish any significant detectable antibodies, which could not be detected with the rSPAG-1 (C-terminus) in any of the calves at any time during the experiment (Fig 4A–D). Increasing the concentration of the protein in ELISA also did not yield any results. The inability of the rSPAG-1 (C-terminus) to detect antibodies may be due to the fact that the protein being expressed only in the sporozoite stage of the parasite, the short pre-lymphocytic period might not be enough to induce any detectable antibodies in the bovine calves during a primary infection. It has also been observed that calves exhibited poor antibody response to sporozoites of *T. parva* following a single immunisation by infection and treatment method; however, effective antibodies to sporozoites were detected in calves after repeated challenge with live

sporozoites (Musoke *et al.* 1982). These findings are in similar line with those of Katende *et al.* (1998) who recorded high antibody titre with recombinant polymorphic immunodominant molecule (PIM), a schizont antigen where as full length recombinant p 67; a sporozoite antigen, gave only low antibody titres.

The *rTaSP* was then used to screen 50 field cattle sera collected from endemic region of the country with crude piroplasmic antigen, which presently serves as the reference antigen in our laboratory. In the present study, *rTaSP* ELISA detected more number of positive sera in the field samples than the soluble piroplasm antigen. Out of the total 50 sera samples screened, 10 sera tested positive with crude piroplasmic antigen while 14 sera were found positive with *rTaSP* for *Theileria* antibodies (Figs 5, 6). All the samples which showed positive reaction with piroplasm antigen were found positive with *rTaSP*. To check the cross reactivity, 3 samples each from animals experimentally infected with *B. bigemina* and *T. evansi* were included in the study. *rTaSP* did not show any cross reactivity with either of the parasite. On the basis of the present study, the *rTaSP* was found to be of having immense potential for early serodiagnosis of bovine tropical theileriosis and if used in combination with merozoite antigens, it could serve as an efficient diagnostic system.

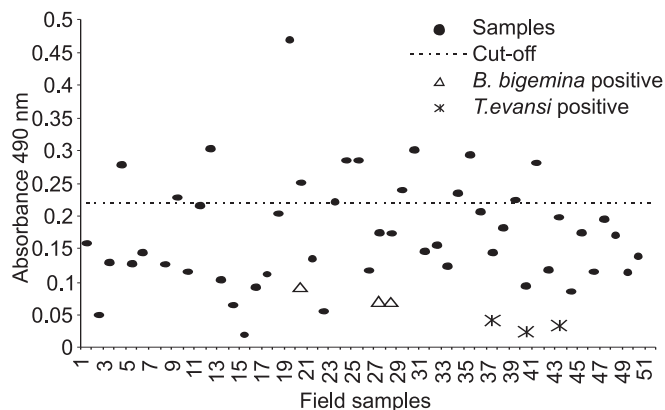


Fig. 5. ELISA O.D distribution of 50 field cattle sera against rTaSP.

This antigen could also be very useful to detect the rate of seroconversion in herds vaccinated with attenuated schizonts of *T. annulata* wherein the piroplasms are not expected to appear in circulation.

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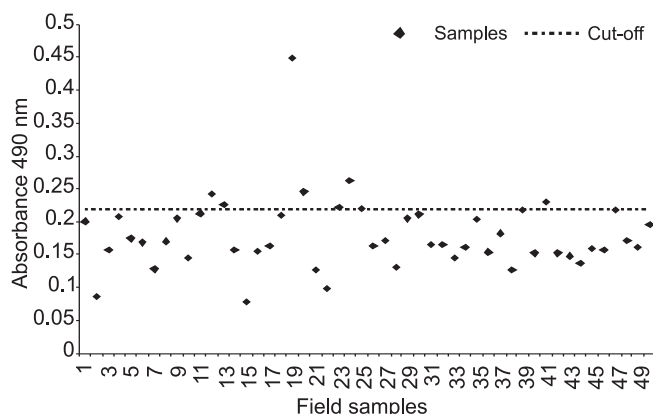


Fig. 6. ELISA O.D distribution of 50 field cattle sera against crude piroplasmic extract.

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