



An inhibition enzyme immuno assay exploring recombinant invariant surface glycoprotein and monoclonal antibodies for surveillance of surra in animals



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ABSTRACT

The present study is aimed at the development of inhibition ELISA (I-ELISA) exploring monoclonal antibodies (MAbs) and recombinant invariant surface glycoprotein. The extracellular domain (ED) of invariant surface glycoprotein (ISG-75) from *Trypanosoma evansi* has been heterologously expressed in *Pichia pastoris* (X-33). The recombinant ISG-75 (rISG-75ED) was characterized by immunoblot and ELISA, followed by the production of MAbs against rISG-75ED. The MAbs were characterized by immunoblot and then explored in the development of I-ELISA for the detection of surra. The diagnostic potential of the developed test has been evaluated using 1192 field sera sample including cattle, buffalo, donkey, horse and camel. The statistical analysis of the data showed optimum combination of diagnostic sensitivity and specificity at 98.8% and 99.2% respectively, with cut-off percentage inhibition (PI) value of >45. The Cohen's kappa coefficient of agreement was found to be 0.98. Hence, the diagnostic test developed in the present study can be exploited as a potential and reliable tool in the serodiagnosis and surveillance of surra in animals.

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1. Introduction

Different species of trypanosomes causes a disease called trypanosomiasis in animals and humans which is endemic throughout Asia, America (central and south), Africa and Europe. *Trypanosoma evansi* a haemoflagellate parasite and believed to be originated from *T. brucei* [1] is an etiological agent of a disease called surra in animals. Surra is considered as an important, chronic wasting disease of domestic and wild herbivores and carnivores in tropical and sub-tropical countries. Moreover, the trypanosomes which are considered as non-infective to humans have emerged as potentially pathogenic to humans. A typical human trypanosomiasis caused by different species of trypanosomes including *T. evansi*, *T. lewisi*, *T. congolense* and many more, have been reported by several groups [2–4]. The clinical symptoms of infected animals include severe anemia, fever, weight loss, hypoglycaemia, poor body weight gain,

poor draughtability, infertility, abortion and may also lead to death in the untreated condition. In the high risk areas of infection there has been a significant reduction in the calving rate in cows and fertility rate in infected bulls [5]. Meanwhile, the buffaloes [6] and cattle [7,8] infected with *T. evansi* have been reported with reproductive problems such as, stillbirth and abortions. Moreover, in lactating cows the significant decrease in milk production has also been observed during the sub clinical stage of the disease [9]. The most susceptible hosts for surra in south-east Asia are cattle, buffaloes, camels and horses [10]. Buffaloes and cattle act as the main parasite reservoir hosts for *T. evansi* [11]. Laha and Sasmal [12] reported a high percentage (12.74%) of *T. evansi* infection in horses from India. Surra is mainly transmitted mechanically by the tabanid flies, however in carnivores the disease transmission has been observed after feeding on infected meat [13].

The detection of the carrier status and subsequent treatment of animals lead to effective control of the disease, as well as better production. Several diagnostic tests (serological and parasitological) have been developed for detection of surra. However, only clinical stages of infections can be diagnosed satisfactorily by parasitological test, but not latent or chronic infection [14]. The serological test such as, ELISA is likely to precisely identify healthy

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animals and qualifies as a universal test [15]. However, the development of molecular diagnostic tools have given a break through in the detection of parasitic diseases. The carrier status of the infection can be detected effectively through detection of parasite nucleic acid [16,17] or by the development of sensitive and specific serological tests. Several genes of trypanosomes have been expressed heterologously for many purposes for instance, variable surface glycoprotein (VSG) gene [18–20], beta-tubulin gene [21], actin gene [22] and ISG-75 gene [23,24].

The blood stream stage specific surface glycoproteins such as, ISGs are uniformly distributed over the entire surface of the trypanosomes [25]. The bloodstream form trypanosomes contain an estimated 5×10^4 ISG-75 molecules on the surface [26], which do not exhibit antigenic variation unlike VSG [27]. Several bloodstream forms of ISGs have been identified and characterized which include ISG-75 [28], ISG-65 or ISG-70 [29], ISG-64 [30], and ISG 100 [25]. The ISG-75 is conserved among all the species and subspecies of the *Trypanozoon* subgenus including *T. evansi*, *T. b. gambiense*, *T. b. rhodesiense* and *T. equiperdum* [26]. Moreover, ISG-75 is found as multiple copies in trypanosomes [31].

The proteins which are invariant and not subjected to antigenic variation such as ISG-75, represent promising and perspective alternatives in the development of diagnostic tests [32]. Hence, in the present study, the monoclonal antibodies were produced against recombinant ISG-75ED and explored in the development of antibody detecting inhibition ELISA (I-ELISA) for the detection of surra. Further, the developed assay was compared with CATT/*T. evansi* using a panel of serum samples collected from different states of India.

2. Materials and methods

2.1. rISG-75ED, immune serum and field serum samples

The ED region of ISG-75 from *T. evansi* expressed in *P. pastoris* [24] was used in the present study for the production of MAbs and development of I-ELISA. In brief, the ED region of ISG-75 from *T. evansi* encoding a polypeptide of 440 amino acids has been cloned into pPICZ α (A) vector and expressed in eukaryotic host such as *P. pastoris* (X-33). The expressed protein (rISG-75ED) was purified and characterized by both immunoblot and ELISA. Further, the diagnostic potential of rISG-75ED in ELISA was determined by panel of field sera sample. The rISG-75ED with an apparent molecular weight of 70 kDa was found to be highly immunoreactive with panel of sera sample [24]. The diagnostic sensitivity and specificity of rISG-75ED in ELISA was found to be 98.47% and 99.70% respectively at >0.547 cut off OD value [24]. The control antigens purified from induced X-33, X-33 with pPICZ α (A) and uninduced X-33 with pPICZ α (A) ISG-75ED were also used in the characterization of rISG-75ED [24] and MAbs.

The immune serum produced against buffalo isolate of *T. evansi* [18] and available in our laboratory was used in the standardization of inhibition enzyme immuno assay. The field serum samples were collected from different states of India from different species of animals including cattle (417), buffalo (172), horse (98), donkey (87), and camel (418). All the serum samples were screened for surra by CATT/*T. evansi* and I-ELISA in duplicate and comparative analysis was done between the two tests (Table 1). The CATT/*T. evansi* kit containing freeze dried *T. evansi* VSG RoTat 1.2 [33,34] was procured from Koning Leopold Institute of Tropical Medicine, Antwerp, Belgium (OIE reference laboratory of surra) and was used as a standard reference test. The field serum samples were tested in CATT/*T. evansi* as per the protocol mentioned earlier by Rudramurthy et al. [23] and in I-ELISA as per the protocol mentioned in section 2.4.

2.2. Production of MAbs

Hybridoma clones producing ISG-75ED specific antibodies were developed by fusing splenocytes and B cell myeloma cells, SP2/O [35]. The splenocytes for fusion were isolated from BALB/c mice (8–12 weeks old), immunized with rISG-75ED. The peritoneal exudate enriched with macrophages was used as a feeder layer for the growth of hybridoma. The splenocytes, SP2/O and macrophages (feeder layer) were prepared by following the protocol described earlier [36]. The sera collected from the immunized and non-immunized BALB/c mice were used as positive and negative controls respectively.

The splenocytes and SP2/O cells were fused by following the protocol mentioned earlier [36]. In brief, for the production of hybridoma, 20 million splenocytes and 2 million SP2/O cells were subjected to fusion using polyethylene glycol (PEG) and dimethyl sulphoxide (DMSO) mixture (Sigma Aldrich). The fused cells were incubated in the CO₂ (5%) incubator for 1½h and then pelleted at $214 \times g$ for 10 min at room temperature. The cell pellet was suspended in IMDM, containing 0.1 mM sodium hypoxanthine (H), 0.4 μ M aminopterin (A) and 0.016 mM thymidine (T) (Gibco®Life technologies), 20% fetal calf serum (FCS) and 20 mM L-glutamate. The hybridoma clones were then screened by indirect ELISA using rISG-75ED (3 μ g/well). The monoclones were selected from the positive hybridoma clones by dilution cloning technique. The hybridoma cells were seeded in the microtiter plates at 10% plating efficiency to give rise to a statistical probability of one colony/well. The monoclones were screened by indirect ELISA, expanded further and preserved till further use. The MAb was then produced in larger scale in 250 mL culture flasks. The culture rich in MAbs (>1.2 OD in indirect ELISA) was centrifuged at 3000 rpm for 3 min to remove the cells and MAbs were preserved at -80°C till further use. The control clones (SP2/O, spleen and macrophage) and positive and negative sera were used during the selection of positive hybridoma and monoclones.

2.3. Characterization of MAbs by immunoblot and isotyping

The immunoblot analysis was carried out to characterize the MAbs by following the standard protocol [37]. In brief, the electrophoresed proteins (rISG-75ED and control antigens) were transferred on to nitrocellulose membrane by following the standard protocol and then the membrane was treated with culture supernatant from monoclones (MAbs) and SP2/O clones (undiluted). The substrate solution containing diaminobenzidine tetrahydrochloride (DAB) was added finally to develop the immunoblot.

Mouse MAbs produced in the present study were isotyped using mouse isotyping (IgG1, IgG2a, IgG2b, IgG3, IgA and IgM) antibodies (Sigma Aldrich) by antigen mediated ELISA by following the protocol mentioned earlier [36].

2.4. Development of I-ELISA

Microtiter plates (Maxisorp®, Nunc) were coated overnight at 4°C with (100 μ L/well) purified rISG-75ED (6 μ g/well) antigen in PBS (pH 7.2). After overnight incubation the microtiter plates were washed and blocked as per the standard protocol. The test serum samples (20 μ L of test serum, 20 μ L of blocking buffer and 60 μ L of MAbs) were added in duplicate and incubated at 37°C for 1h on ELISA shaker followed by washing. Negative, strongly positive (>90% inhibition), weakly positive (60–70% inhibition), MAb (0% inhibition) and conjugate control wells were also run simultaneously. MAb control wells (Cm) contained all the reagents except the test serum, while conjugate control contained only blocking buffer. Hundred microlitre of anti-mouse antibody horseradish

Table 1Comparative analysis of the serological data obtained from I-ELISA and CATT/*T. evansi*.

Test→		I-ELISA		CATT/ <i>T.evansi</i>		Chi square (χ^2) value	Significance (P) value
State	Species	P	N	P	N		
Karnataka	Cattle	39	99	40	98	0.018	0.89
	Buffalo	56	116	54	118	0.053	0.81
	Horse	5	93	5	93	0.0	1.0
	Donkey	6	81	6	81	0.0	1.0
Odisha	Cattle	6	34	6	34	0.0	1.0
West Bengal	Cattle	92	147	92	147	0.0	1.0
Rajasthan	Camel	95	323	94	324	0.007	0.93

peroxidase conjugate was diluted as per the manufacturer's instruction (Bethyl Laboratories) and added to each well. Later the plates were incubated at 37 °C for 1h followed by washing and addition of substrate (O-Phenylene Diamine) solution (100 μ L/well). The plates were incubated till the visible color develops in the Cm wells and then the reaction was stopped by the addition of (100 μ L/well) stopping solution. The plates were then read at 492 nm in an ELISA reader. The OD values obtained were converted to percentage inhibition (PI) values by using the formula: $PI = 100 - \{[(OD \text{ in test well} \div OD \text{ in Cm well}) \times 100]\}$ [38]. The optimum antigen concentration, MAb and test serum dilutions were determined by checker board titrations.

2.4.1. Determination of diagnostic sensitivity and specificity of I-ELISA

The cut-off PI, diagnostic sensitivity and specificity were determined using field serum samples. The field serum samples were subjected to I-ELISA in duplicate and average OD values obtained were converted to PI. The data was subjected to receiver operating characteristic (ROC) analysis by keeping CATT/*T. evansi* as a gold standard test. Software MedCalc, version 12.7.2 (<http://www.medcalc.org>) was used to generate ROC curves and area under curve (AUC). The specificity of I-ELISA was evaluated with cattle serum samples clinically infected with *Theileria annulata* and *Babesia bigemina*. Furthermore, the significance of the new assay was determined by Chi square (χ^2) analysis [39] of the data (species wise).

3. Results

3.1. Production and characterization of MAbs

We were able to generate three monoclonal clones out of ten hybridoma clones and were named MISG121, and MISG122. The MAbs (MISG121 and MISG122) were found to be immunoreactive and a band corresponding to 70 kDa revealed that the MAbs are highly specific to rISG-75ED (Fig. 1). However, the immunoreactivity of MAbs was not observed with control proteins (Fig. 1). Isotyping analysis revealed that, both MAbs (MISG121 and MISG122) belong to isotype of IgG, subtype of IgG2b. MISG122 showed highest reactivity with rISG-75ED in indirect ELISA (OD > 1.4) compared to MISG121. Hence, MISG122 has been explored in the development of I-ELISA in the present study. While, the control clones (SP2/O, spleen and macrophages) showed lesser OD values (<0.4).

3.2. Diagnostic sensitivity and specificity of I-ELISA

Based on the checker board titration, the optimum concentration of antigen (rISG-75ED), buffalo immune serum and MISG122 were found to be 6 μ g/well, 20 μ L/well and 60 μ L/well respectively. The statistical analysis of the data showed positive–negative cut-off PI value at >45. The diagnostic sensitivity and specificity was

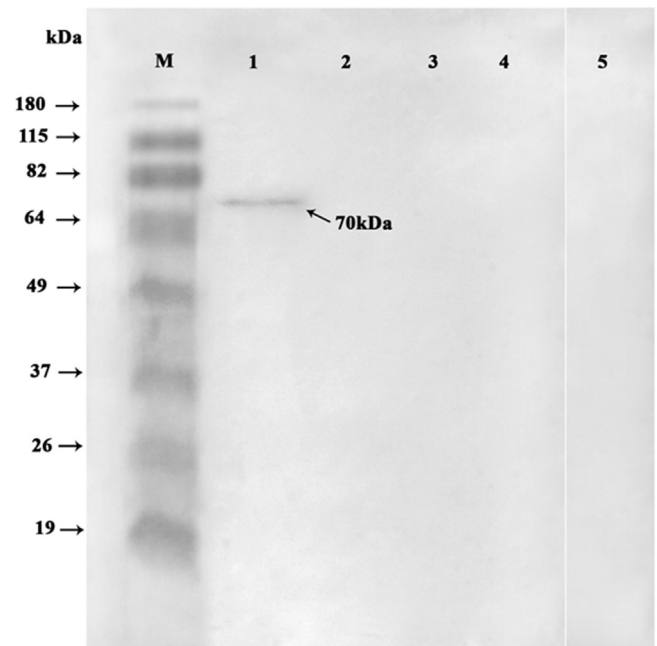


Fig. 1. Immunoblot analysis of MAbs: Lane M = prestained protein ladder, lanes 1 to 4 treated with MAb, lane 5 treated with SP2/O culture supernatant, lanes 1 and 5 = rISG-75EDek, lanes 2, 3 and 4 = purified proteins from induced X-33, induced X-33 with pPICZ α (A), uninduced X-33 with pPICZ α (A)+ISG-75ED respectively.

found to be respectively at 98.8% [95% confidence interval (CI), 97.2 to 99.6]) and 99.2% (95% CI, 98.2 to 99.7) (Fig. 2). The Cohen's kappa coefficient of agreement value between I-ELISA and CATT/*T. evansi* was found to be 0.98. The Chi square (χ^2) analysis also revealed no significant difference between I-ELISA and CATT/*T. evansi* (Table 1). Hence, the developed assay is in agreement with the standard reference test for surra. Moreover, the cattle sera sample clinically infected with *Theileria annulata* and *Babesia bigemina* were found to be negative in I-ELISA.

4. Discussion

Different species of trypanosomes such as *T. evansi*, *T. equiperdum* and *T. brucei* sp., causes a chronic wasting disease called trypanosomiasis. Trypanosomiasis is considered as the neglected tropical disease in domestic live stock; but causes a significant economic loss in agricultural industry. The detection and subsequent treatment are the effective measures to control the disease. The development of non-cross reactive tests plays a significant role in the serodiagnosis of surra. Moreover, the proteins which are unique to trypanosomes and are invariant in a range of different isolates/strains of the pathogen play a pivotal role in the serodiagnosis [32].

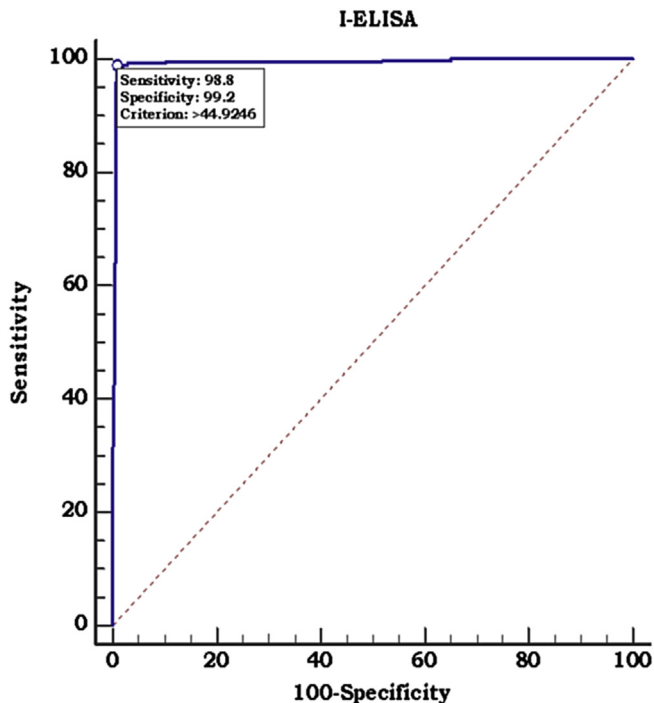


Fig. 2. ROC curve depicting the diagnostic sensitivity and specificity of inhibition ELISA.

In the present study, the monoclonal antibodies were produced against rISG-75ED of *T. evansi*. The isotyping analysis revealed that the MAbs belong to isotype IgG, subtype of IgG2b. Immunoblot assay revealed the higher immunoreactivity and specificity of MAbs to ISG-75 of *T. evansi*. Further, the immune serum raised against *T. evansi* was also reactive in I-ELISA. The statistical analysis of the data revealed that, I-ELISA exploring MISG122 has diagnostic sensitivity of 98.8% and specificity of 99.2%, with Cohen's kappa coefficient of agreement value 0.98. The non-reactivity of sera sample clinically infected with *Theileria annulata* and *Babesia bigemina* in I-ELISA revealed that the test is specific to trypanosomes. Moreover, Chi square (χ^2) analysis of the data obtained from reference test and new assay showed no significant difference (Table 1). Hence, the new assay developed in the present study turns out to be an accurate diagnostic tool for surra.

Several groups have successfully produced monoclonal antibodies against different antigens of trypanosomes e. g. against invariant antigens of vector forms of *Trypanosoma simiae* [40], against metacyclic trypomastigotes stage of *Trypanosoma cruzi* [41], associated antigens against *Trypanosoma cruzi* [42] and against a flagellar antigen of *T. evansi* [36,43]. Heterologously expressed proteins (recombinant proteins) play significant role in the development of diagnostic tests. The recent study by Sengupta et al. [44], had shown the diagnostic potential of VSG from *T. evansi* expressed in *P. pastoris*. Moreover, the ELISAs based on MAbs and recombinant antigens have tremendous potential/application in the diagnosis of parasitic diseases. I-ELISA can detect carrier animals and also can be used with ease using recombinant antigens [45]. Since, MAbs antibodies are raised against a single epitope; the specificity achieved by I-ELISA can be very high and is an organism specific. By using single anti-mouse immunoglobulin conjugate, serum from any animal species can be tested with shorter turnaround time and lower expense. Hence, I-ELISA has an additional advantage over those of conventional serologic assays and monoclonal antibodies act as potent reagents in the diagnosis of diseases.

5. Conclusion

The emergence of atypical human trypanosomiasis due to *T. evansi* is drawing a great attention from the medical field. As per the literature search this is the first ever endeavour of producing monoclonal antibody against invariant surface glycoprotein of *T. evansi*. Moreover, it is well established that ISG-75 is highly conserved among different species of trypanosomes such as, *T. evansi*, *T. equiperdum* and *T. brucei* and also genus specific rather than species specific. Hence, the present finding can be exploited as a reliable, potential and promising perspective tool for the future application in the serodiagnosis of trypanosomiasis in animals as well as humans. However, further study may help in the application of new assay in the diagnosis of atypical human trypanosomiasis and also trypanosomiasis caused by different species in animals.

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