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Short communication

Development of an antigen ELISA using monoclonal antibodies against recombinant VSG for the detection of active infections of *Trypanosoma evansi* in animals

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ABSTRACT

Trypanosoma evansi, a haemo-flagellated protozoan parasite causes chronic wasting disease in a wide range of animals. For its diagnosis, blood smear examination is useful in clinical cases for direct identification of the parasite but in latent infection the carrier animals are difficult to screen out by conventional blood smear test. Harboring low level of parasites and showing no symptom, the carrier animals for surra can act as a source of infection. The level of parasitaemia fluctuates, especially during latent infection; moreover the antibodies which are not found early in the infection may persist even after recovery or chemotherapy. In the present study a double antibody sandwich ELISA exploring, monoclonal antibodies and hyperimmune serum, raised against recombinant variable surface glycoprotein has been developed to detect circulating trypanosome antigens. The developed antigen detection ELISA (Ag-ELISA) was evaluated using 652 blood samples collected from cattle, buffalo, equine and camel. The statistical analysis of the data showed diagnostic sensitivity and specificity at 97.4% and 96.4% respectively, with a positive-negative cut-off OD value > 0.28. Furthermore, the detection limit of the assay was found to 7.15 trypanosomes per mL. The present finding revealed that the developed assay can be exploited as a potential diagnostic test in the detection of circulating trypanosome antigens and also can be used as a population screening test for multiple animal species for detection of active infection for further treatment and control of the disease.

1. Introduction

Trypanosoma evansi, a petite mutant of *T. brucei* (Field and Carrington, 2009) is a haemoflagellated parasite, which causes a fatal wasting disease known as "surra". Surra has a broad range of hosts such as cattle, buffaloes, mules, donkeys, horses, camels *etc.* and results in production loss. In South east Asia cattle, buffaloes and horses are the most common mammalian hosts for surra (Holland et al., 2004). The clinical symptoms of the disease are anemia, recurrent fever, muscular weakness, oedema, loss of appetite and abortion. After recovery of the disease, animals exhibit low levels of fluctuating parasitaemia for years and thus serve as carriers for the disease. Hence, the detection of carrier status is very significant in controlling the disease. Several potential serological tests targeting surface antigens have been developed to detect either antigens or antibodies such as, antigen detection ELISA for camels (Diall et al., 1992; Verloo et al., 2000), buffaloes (Davison et al., 1999; Verloo et al., 2000), antibody detection based on the predominant variant antigen type (VAT) RoTat 1.2 (Verloo et al., 2000), CATT/*T. evansi* (Bajyana Songa and Hammers, 1988; Verloo et al., 2000), LATEX/*T. evansi* (Verloo et al., 1998, 2000) and ELISA/*T. evansi* (Verloo et al., 2000). Furthermore, several techniques such as, DNA amplification and serological tests have been developed targeting many genes such as, variable surface glycoprotein (VSG) (Sengupta et al., 2010, 2012, 2014; 2016, 2018), invariant surface glycoprotein (ISG) (Rudramurthy et al., 2013, 2015, 2017a, 2017b, 2018), nuclear DNA, kinetoplastid DNA and multilocus isoenzymes analysis (Stevens and Godfrey, 1992; Zhang and Battz, 1994; Mathieu-Daude and Tibayranc, 1994).

The conventional parasitological techniques, which are most commonly practiced at field level can satisfactorily diagnose acute or sub acute infections, but more difficult in latent or chronic infection. In this regard, there is a need to develop sensitive and specific diagnostic techniques.

VSG is the primary and major immunogen of *T.evansi* in eliciting the

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antibody response in host and is uniformly distributed over the entire surface of trypanosomes. Several groups have successfully expressed different genes of trypanosomes for several purpose in different host cells such as, VSG (Urakawa et al., 2001; Sengupta et al., 2012, 2014; 2016), acid α-mannosidase and trans-sialidase from *T. cruzi* (Laroy and Contreras, 2002; Vandersall-Nairn et al., 1998), rhodesain from *T. brucei rhodesiense* (Caffrey et al., 2001) and congopain from *T. congolense* (Huson et al., 2009). Moreover, monoclonal antibodies (MAbs) have been generated by several groups against different antigens of trypanosomes such as, invariant antigens, metacyclic trypomastigotes stage, associated antigens and many more (Manas et al., 1986; Takasu et al., 1989; Bosompem et al., 1995) and the developed MAbs have been characterized for the development of antigen detection ELISA.

Hence, in the present study a new double antibody sandwich ELISA has been developed exploring MAbs produced against recombinant VSG (rVSG). The developed assay has been characterized with panel of field samples from cattle, buffalo, equine and camel to determine the sensitivity and specificity of the developed assay.

2. Materials and methods

2.1. *T. evansi* stabilates and experimental animals

T. evansi isolated from buffalo, in India (Karnataka state) and maintained in liquid nitrogen at Parasitology laboratory NIVEDI, Bengaluru, India was used in the present study. The parasite was propagated *in-vivo* in albino rats and at peak parasitaemia the rats were sacrificed and the parasites were isolated from the buffy coat by following the protocols mentioned earlier (Sengupta et al., 2010; Rudramurthy et al., 2013). All the experimental animals of the present study were treated as per the standard protocols of animal ethics and the institute's registration number is 881/CPCSEA. Feed and drinking water were given *ad libitum*.

2.2. rVSG, MAbs hyperimmune serum and field samples

The VSG gene from *T. evansi* expressed in *Pichia pastoris* (X-33) has been used in the production of MAbs. The expressed VSG protein (rVSG) having a molecular weight of ˜68 kDa, has been explored in the development of antibody detecting ELISA. And this revealed the diagnostic sensitivity and specificity of 95.4% and 93.8% respectively with a cut off OD value of > 0.495 (Sengupta et al., 2016). Further, the MAbs were developed against rVSG and characterized by immunoblot and isotyping analysis as per the standardized protocol (Ligi et al., 2016; Sengupta et al., 2018). Two monoclonal hybridoma clones generated against rVSG (MVSG121 and MVSG122 were found to be immunoreactive and belong to isotype of IgG, subtype IgG3. Out of these two clones, the MVSG121exhibited higher reactivity with rVSG compared with MVSG122. Furthermore, the MVSG121 has been explored in the development of antibody detecting competition ELISA and the diagnostic sensitivity and specificity was found to be 92.6% and 96.4% respectively, at > 40 percent inhibition (Sengupta et al., 2018).

The hyper immune serum raised against rVSG and preserved at −80 °C (Sengupta et al., 2016) was used in the development and characterization of Ag-ELISA. The blood samples were collected (with anticoagulant, Heparin) from cattle (n = 181), buffalo (n = 197), camel (n = 176) and horse (n = 98) from Karnataka and Rajasthan states of India and preserved at 4 °C till further use. All the blood samples were then subjected to newly developed assay (Ag-ELISA) and diagnostic PCR in duplicate. The diagnostic PCR was carried out as per the protocol mentioned by Sengupta et al. (2010). In brief the DNA was isolated from all the blood samples and subjected to PCR using DITRY-F/R primers. The 50 μL reaction mixture contained 100 ng genomic DNA, 10 mM Tris HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 100 mM each of four dNTPs, 10 pmol each of the oligonucleotide primers (DITRYF/R) and 1.0 U Taq DNA polymerase (Fermentas). The amplification was carried out with an initial denaturation at 94 °C for 3 min (min) followed by, 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 54 °C for 1 min and primer extension at 72 °C for 1 min. The final primer extension was carried out at 72 °C for 10 min. The amplified products were run on 1% agarose gel and then observed 400 bp amplified DNA under UV Transilluminator.

2.3. Development of Ag-ELISA

For Ag-ELISA, the microtiter plates (Maxisorp®, Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 100 μL/well of MVSG121 (undiluted culture supernatant). After overnight incubation the plates were washed and blocked as mentioned earlier (Sengupta et al., 2012). After washing the plates, the blood/buffy coat/purified trypanosomes samples (blood samples from field animals and buffy coat from experimentally infected rats) were added (100 μL/well) in duplicate and incubated at 37 °C for 1 h on shaker followed by washing. Negative (healthy blood/buffy coat samples), positive (truly positive blood/buffy coat samples) and conjugate control (absence of competing MAbs) wells were also run simultaneously. One hundred microlitre of rabbit hyperimmune serum raised against rVSG was added to each well, followed by incubation and washing. After washing, 100 μL of anti-rabbit antibody horseradish peroxidase conjugate (diluted as per manufacturer's instruction) was added to each well and incubated, followed by washing and addition of substrate solution (100 μL/well). The substrate solution was prepared by dissolving 5 mg of o-phenylenediamine dihydrochloride (Sigma) and H_2O_2 (0.03%), in phosphate citrate buffer (Na_2HPO_4 (0.2 M), citric acid (0.1 M), pH 5.0). The plates were incubated till the development of color and the reaction was then stopped by the addition of (100 μL/well) stopping solution (1 M H_2SO_4). The plates were then read at 492 nm in an ELISA reader.

2.4. Determination of diagnostic sensitivity and specificity of Ag-ELISA

The optimum combination of diagnostic sensitivity and specificity of Ag-ELISA was determined using the data by keeping diagnostic PCR as a gold standard test. The statistical analysis was done through receiver operating characteristic analysis as per Sengupta et al. (2016). The specificity of Ag-ELISA was also evaluated with cattle blood samples (two each) clinically infected with *Theileria annulata* and *Babesia bigemina.* The agreement between the two antigen detecting tests was determined by Chi square ($\chi 2$) analysis (Snedecor and Cochran, 1968) of the data.

2.5. Determination of analytic sensitivity of Ag-ELISA by dilution assay

The analytic sensitivity of Ag-ELISA was also evaluated with rat blood and buffy coat samples experimentally infected with *T. evansi* and also with purified trypanosomes. In brief, at peak parasitemia the rats infected with *T. evansi* were sacrificed (Sengupta et al., 2010; Rudramurthy et al., 2013) to collect blood and buffy coat and preserved at 4 °C till further use. The trypanosomes were purified from the buffy coat (Lanham and Godfrey, 1970) and stored at 4 °C till further use. The trypanosomes in blood, buffy coat and purified samples were enumerated using haemocytometer. The blood and buffy coat samples were diluted (1:1) respectively with apparently healthy rat blood and buffy coat, while purified trypanosomes were diluted (1:1) with sterile phosphate buffered saline (PBS). Each dilution was subjected then to Ag-ELISA (duplicate) and the maximum dilution at which, the OD value is above the cut-off OD value was selected as a sensitivity level and expressed as number of trypanosomes/mL of blood/buffy coat/purified sample.

Table 1
The comparative Chi square analysis of diagnostic PCR and Ag-ELISA for seropositivity of surra.

State	Species	Ag-ELISA		Diagnostic PCR		Chi square (χ2) Value	Significance (P-value)
		P	N	P	N		
Karnataka	Cattle	39	142	40	141	0.016	0.8987
	Buffalo	48	149	48	149	0.000	1.0000
	Horse	5	93	5	93	0.000	1.0000
Rajasthan	Camel	56	120	53	123	0.120	0.7295

P: Positive, **N:** Negative, **PCR:** Polymerase chain reaction, **Ag-ELISA:** Antigen detection ELISA.

3. Results

3.1. Diagnostic sensitivity and specificity of Ag-ELISA

The ROC analysis revealed the highest combination of diagnostic sensitivity and specificity of 97.4% [95% confidence interval (CI), 95.5–99.7] and 96.4% (95% CI, 94.6–98.5) respectively with a cut off OD value > 0.28. The Cohen's kappa coefficient of agreement value was found to be 0.93. The positive and negative predictive values are respectively 96.4 and 97.4 respectively with a classification accuracy of 96.9. Moreover, the reactivity of cattle blood samples clinically infected with *Theileria annulata* and *Babesia bigemina* was not found in Ag-ELISA and diagnostic PCR. The Chi square (χ2) analysis revealed that the tests are in agreement with each other (Table 1).

3.2. Determination of analytic sensitivity of Ag-ELISA by dilution assay

Moreover, the evaluation of sensitivity by dilution of *T. evansi* infected rat blood, buffy coat and purified samples showed that, the Ag-ELISA detected 7.15 trypanosomes per mL from blood, buffy coat and purified sample (Fig. 1). The OD values obtained from blood, buffy coat and purified trypanosomes samples at this cut off value is different and purified trypanosomes samples had shown higher OD value followed by buffy coat and blood. Furthermore, though, OD value at 3.57 trypanosomes per mL is greater than the cut off OD value, 7.15 trypanosomes per mL is considered as the sensitivity level to eliminate any false negative/positive results which appear due to error in parasite enumeration.

3.3. Comparative analysis of seropositivity of field samples

Out of 181 cattle field samples, 39 samples were found to be positive by Ag-ELISA, while 40 samples were found positive by PCR. The overall seropositivity of cattle for trypanosomosis in Karnataka state of India was observed as 21.5% and 22% by Ag-ELISA and PCR respectively. While, 48 buffalo samples out of 197 were found positive by both Ag-ELISA and PCR, with a seropositivity of 24.3%. The trypanosomosis in camel was found to be 31.8% by Ag-ELISA and 30.1% by PCR from Rajasthan state of India (Table 1). However, horse sera sample showed very low prevalence of 5.1% positivity.

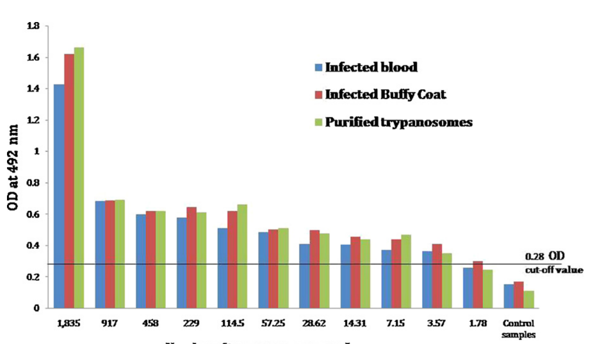

Fig. 1. Determination of analytic sensitivity of Ag-ELISA through dilution assay; Blood and buffy coat samples from the *T. evansi* infected rat and purified *T. evansi* were diluted in respective healthy blood, buffy coat and PBS through 1:1 dilution. The control samples include blood and buffy coat from healthy rat and sterile phosphate buffered saline.

4. Discussion

A wide range of trypanosomes species such as, *T. evansi, T. equiperdum* and *T. brucei* sp., causes a chronic wasting disease. Several serological tests have been developed for the detection and effective control of the disease, for instance, Suratex (Nantulya, 1994), CATT/*T. evansi* (Bajyana Songa and Hammers, 1988), LATEX/*T. evansi* and ELISA/ *T. evansi* (Verloo et al., 2000). It is well known fact that the circulating antigen remained in the chronic/carrier status of the disease and parasite in the blood is not detectable by the conventional methods of diagnostic test. The parasitological test satisfactorily diagnoses clinical stages of infection, than latent or chronic stage (Fernandez et al., 2009) and also exhibits low sensitivity (Luckins, 1992). Hence, the development of highly sensitive and specific test is needed to detect low level of parasitemia in animals including carriers. Surra being an endemic disease, antigen detection is of more implicative and indicative toward the presence of active infection rather than antibody. After acquiring infection the antibody may persist up to 6 month (Yadav et al., 2014). It is well known that, after the treatment for surra the animal becomes infection free but the antibodies remain for prolonged periods. Therefore, simple detection of antibody may not reveal the presence of infection (organism). In such cases, a mass screening antigen detection test is very useful to detect the active infection and the positive animals may be taken for treatment which leads to a very strong control programme. In the present study we recorded a very high sensitivity and specificity, which would enable the test to become useful in screening field animals.

A double antibody sandwich ELISA has been developed in the present study, exploring MAbs and hyperimmune serum produced against recombinant rVSG. The statistical analysis of the data revealed that Ag-ELISA has sensitivity of 97.4% and specificity of 96.4%. Moreover, the Cohen's kappa coefficient of agreement was found to be 0.93 with a classification accuracy of 96.9. Besides, the blood samples clinically infected with *Theileria annulata* and *Babesia bigemina* did not react in Ag-ELISA. Hence, Ag-ELISA exploring MAbs is providing a possible diagnostic tool for the detection of trypanosome antigens in different species of animals. Earlier, our group has developed and characterized indirect ELISA, competitive ELISA, inhibition ELISA and antigen detection ELISA using respective recombinant VSG and ISG antigens and corresponding MAbs. The inhibition ELISA exploring MAbs generated against recombinant ISG had shown sensitivity and specificity of 98.8% and 99.2% respectively (Rudramurthy et al., 2017b). Whereas, competitive ELISA exploring MAbs generated against recombinant VSG had shown sensitivity and specificity of 92.6% and 96.4% respectively (Sengupta et al., 2018). Furthermore, antigen detection ELISA developed by our group exploring MAbs against ISG also showed high sensitivity (97.4%) and specificity (99.0%) with a detection limit of 11.5 *Trypanosoma evansi* per mL (Rudramurthy et al., 2018). The present study falls in line with the previous studies having on par diagnostic sensitivity and specificity. However, the detection limit of the present assay is detected 7.15 trypanosomes per mL, which is little higher than the previous study. Though, there is a small difference in the sensitivity and specificity values between VSG and ISG antigens, both the antigens qualifies as a potential diagnostic tools. Hence, the different ELISAs developed by our group exploring recombinant VSG and ISG and their corresponding MAbs can be used at the field level depending upon the availability of the test.

The study of animal trypanosomosis in Karnataka state of India revealed a positivity of 21.5% and 24.3% in cattle and buffalo, respectively. The survey of camel and horse trypanosomosis had shown a positivity of 31% and 5.1% respectively. The low prevalence of surra in horse may due to the very good management practices followed in the stud farm. However, a larger panel of samples with heterologous and homologous infections of cattle, buffalo, equines and camels from other parts of India needs to be evaluated in order to provide reliable data on prevalence of the disease. India hosts largest population of cattle and buffalo in the world (DADF, 2007). In many tropical countries like India where tse-tse fly is not available trypanosomosis in animals is caused due to *T. evansi* infection which causes substantial production losses. So a mass level antigen detecting screening test will be helpful to detect the carrier animal and subsequent treatment for effective control of the disease.

5. Conclusion

Several PCR assays targeting different genes of trypanosomes are available for the detection of trypanosomosis. However, the sensitivity of the PCR is determined by the number of copies of the target gene, DNA preparation step and method of parasite DNA concentration determination. The Ag-ELISA does not depend on any of the above facts and could be easily applied at the field level. Although antigen levels fall rapidly after treatment, antibody levels continue to rise; hence the rapid disappearance of antigen from the circulation confirms the successful treatment of animals and also minimizes false positive results. The developed assay exhibited highest combination of sensitivity and specificity. Hence, the new assay (Ag-ELISA) can be explored as a reliable, potential and promising perspective tool for future applications in the field.

Conflict of interest

There is no conflict of interest between our research data or work with anybody.

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