Diagnosis of *Trypanosoma evansi* in bovines by indirect ELISA

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Abstract The diagnosis of *Trypanosoma evansi* in animals with low parasitaemia is hampered by low diagnostic sensitivity of traditional detection methods. The present study was undertaken with an objective to improve the diagnostic tools for detection of antibodies against *T. evansi* infection using indirect enzyme-linked immunosorbent assay (ELISA) in bovines. The optimum concentration of antigen, test sera and conjugate were determined as 5 µg per well, 1:10 and 1: 6,000 dilutions, respectively. Among 320 cattle and 382 buffaloes examined in different parts of Rayalaseema region of Andhra Pradesh for *T. evansi* infection, 36.12 and 31.87 percent were found positive by indirect ELISA, respectively.

Keywords Indirect ELISA · *Trypanosoma evansi* · Bovines · Diagnosis · Antibodies

Introduction

Trypanosoma evansi is mechanically transmitted by hematophagous insects and affects many tropical regions worldwide. *T. evansi* causes a disease known as ‘surra’ in numerous mammalian hosts. The incidence and the severity of the disease vary with the strain of the parasite as well as the species of host affected (Sivajothi et al. 2013c). The standard laboratory method for diagnosis of trypanosomiasis

is to demonstrate and identify trypanosomes in the blood of the infected animal. There are several techniques for parasite detection, which include direct microscopy, concentration techniques and animal inoculation. Clinical signs in different animals vary based on the severity of the infection (Sivajothi et al. 2013a). The clinical signs are not pathognomonic and the standard techniques for the detection of trypanosomes are not sufficiently sensitive. WBF and stained blood smear examination, the commonly used field tests for detection of *T. evansi*. Animal inoculation methods appear to be more sensitive but are laborious and not useful for immediate diagnosis (Sivajothi et al. 2013b). In the face of these constraints, alternative methods of diagnosis have been developed, most of which are for the detection of antibody responses to the antigens of the infecting trypanosomes. The most useful of these tests, in view of their sensitivity and specificity, are the enzyme immunoassay (ELISA) which is used for the diagnosis of *T. evansi* infections. The ELISA is a test that is both analytically and diagnostically sensitive and diagnostically specific. Interpretation of results can be quantitative and objective and the assay may be fully automated, so that large numbers of sera can be screened (O.I.E. 2004). Studies on the antibody response in bovines infected with South Indian isolate of *T. evansi* are limited; hence the present investigation was designed to detect antibodies against *T. evansi* in bovines by indirect ELISA.

Materials and methods

A cattle isolate of *T. evansi* was maintained in the wistar rats for bulk harvest of parasites. At the high of parasitaemia, the rats were bled by heart puncture and the blood sample was immediately diluted with chilled phosphate

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buffered saline glucose (PBS-G), pH 8.0. The separation and purification of *T. evansi* from infected blood was accomplished by using DEAE (Diethyl amino-ethyl cellulose anion exchange column chromatography), as per method of Lanham and Godfrey (1970) with minor modifications. Height of column is 3.5 cm, when kept in a 20 ml standard glass syringe. Whole cell lysate (WCL) antigen was prepared from purified trypanosomes as described by Singh et al. (1994). The whole cell lysate antigen (WCL Ag) was partially purified after precipitating with 50 percent saturated ammonium sulphate followed by extensive dialysis against PBS, pH 7.4 (Singh et al. 1994). Protein concentration of the WCL Ag of *T. evansi* prepared in the present study was estimated as per Lowry et al. (1951) and it was adjusted to 1.0 mg/ml in PBS, pH 8.0 and stored at -20°C in 1.0 ml aliquots.

Blood samples from naturally infected or suspected cattle and buffaloes were collected from jugular vein in separate vials without EDTA for serum collection. Serum samples were collected in sterile vials. Few drops of 1:10,000 sodium azide solution was added to the serum samples and stored at -20°C until use. The hyper immune sera (HIS) was raised in two healthy New Zealand white rabbits, weighing up to 1.5–2.0 kg body weight against WCL Ag as per the method of Singh and Chhabra (1993) with slight modifications. Antibody levels of HIS raised against WCL Ag was confirmed by agar gel precipitation test and counter immune electrophoresis. Hyper immune sera collected from the immunized rabbits was passed through $0.45\ \mu$ membrane filter, aliquoted in sterile vials (1 ml/vial) to avoid multiple freeze–thaw cycle and stored at -20°C until use and it was used as positive control in standardization of Indirect ELISA for detection of circulatory antibodies of *T. evansi*. Pre immunized serum of these experimental rabbits was also stored at -20°C till use as negative control serum for standardization of Indirect ELISA in the present investigation.

Indirect ELISA was standardized according to Shahardar et al. (2008) with some modifications to detect the *T. evansi* antibodies from 702 serum samples of cattle ($n = 320$) and buffaloes ($n = 382$). The concentrations of the antigen (WCL Ag), test sera and conjugate (rabbit anti bovine IgG-HRPO) for optimum performance of the assay were determined by checker board titration. Flat bottom, high binding capacity 96-well ELISA plates (Nunc, Denmark) were used in the study. Unbound sites in the wells of the plate after antigen coating were blocked with 1 % bovine albumin serum (BSA) in PBS with tween-20 (PBST). Antibody concentration was standardized by using known positive and negative sera. Reaction with anti species antibody—enzyme-conjugate was developed by adding hydrogen peroxide (30 %) in tetra methyl benzidine

(TMB) solution. The substrate/chromogen reaction was stopped by adding 5 M H_2SO_4 /3 N NaOH solutions and the readings were taken immediately in the ELISA reader (Qualigens, India) at 450 nm. Cut-off value of optical density (OD) for the assay was established by mean of the OD values shown by the panel of negative serum samples plus three standard deviations. The sera having OD above the cut-off value were considered as positive. The collected sera were processed for the presence of antibodies of *T. evansi* infection by indirect ELISA. Appropriate positive and negative control sera, antigen control and conjugate control were included in each plate.

Results and discussion

In the present study, the *T. evansi* parasite was isolated from a field case of cattle and was propagated in rats and successfully purified by DEAE-cellulose chromatography. Height of column is 3.5 cm, when kept in a 20 ml standard glass syringe and phosphate buffered saline- glucose (PSG) (5:5) were found the most satisfactory in performance. Singh et al. (1994) reported PSG (6:4) as appropriate buffer for purification of *T. evansi*. Concentration of WCL antigen of *T. evansi* was determined by Lowry's method as 60 mg/ml.

Indirect ELISA was optimized as shown in Table 1 for detection *T. evansi* antibodies in sera samples collected from cattle and buffaloes in Rayalaseema region of Andhra Pradesh. Among the sera samples examined from 320 cattle and 382 buffaloes, 31.87 and 36.12 per cent were found positive for *T. evansi* infection respectively. The optimum concentration of WCL Ag of *T. evansi* for coating the wells of ELISA plate determined in the study as $5\ \mu\text{g/ml}$ is in agreement with findings of Desquesnes et al. (2009) ($5\ \mu\text{g/ml}$) and Shahardar et al. (2008) ($5\ \mu\text{g/ml}$). However, Jithendran et al. (1997) used $10\ \mu\text{g/ml}$ and Verloo et al. (2000) used $2\ \mu\text{g/ml}$ for coating of micro plate wells. The optimum test serum dilution found in the present study was 1:10. However, little higher or lower dilutions reported by previous workers as (1:50) by Jithendran et al. (1997), (1:100) by Desquesnes et al. (2009), (1:200) by Shahardar et al. (2008) and (1:400) by Verloo et al. (2000). The optimum dilution of 1: 6,000 rabbit anti-ovine Ig G-HRPO conjugate used in the study is in agreement with Jithendran et al. (1997). Baghel et al. (1996) found positive in 76.53 per cent buffaloes by indirect ELISA. Sinha et al. (2006) reported *T. evansi* prevalence in 52.69 and 52.31 per cent of cattle and buffaloes in Patna. Molina et al. (2000), Desquesnes et al. (2009) reported 9.0 and 83.3 % positive by Ab- ELISA, respectively .

Table 1 Conditions optimized for indirect ELISA to determine the *T. evansi* antibodies in bovines

S. No.	Steps	Antigen/antibody concentrations	Description of step	Volume (μl/well)
1	Coating with <i>T. evansi</i> WCL antigen	5 μg/ml in 50 mM phosphate buffer, pH 7.0	Over night at 4 °C	100
2	Washing	–	PBST; 3 × 3 min	150
3	Blocking	–	1 % BSA in PBST; 1.5 h at RT	125
4	Washing	–	PBST; 3 × 3 min	150
5	Test sera	Neat and serial dilution	2 h at RT	100
6	Washing	–	PBST; 3 × 3 min	150
7	Anti-species-HRPO conjugate	1:6,000 dil. in PBST	1 h at RT	100
8	Washing	–	PBST; 4 × 3 min Last washing in CAB, pH 5.0	150
9	Substrate	100 μl TMB in 1 ml of CAB, pH 5.9 + 9 ml D.W with 5 μl of 30 % H ₂ O ₂		100
10	Stopping	4 N H ₂ SO ₄		50
11	O.D. values were taken at 450 nm absorbance mode after stopping the reaction			

PBST phosphate buffered saline (pH 7.0) with Tween 20 (0.05 %), RT room temperature, TMB Tetra methyl benzidine (Genei, Bangalore), CAB Citrate-Acetate buffer, pH 5.9

Summary

The present study was undertaken with an objective to improve the diagnostic tools for detection of *T. evansi* infection in bovines. Indirect ELISA was standardized for detecting circulatory antibodies of *T. evansi* in the sera of bovines. The optimum concentration of antigen, test sera and conjugate were determined as 5 μg per well, 1:10 and 1: 6,000 dilutions, respectively. Among 320 cattle and 382 buffaloes examined in different parts of Rayalaseema region of Andhra Pradesh for *T. evansi* infection, 36.12 and 31.87 percent were found positive by indirect ELISA, respectively.

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