Journal of
Advanced Veterinary Research
Volume 4, Issue 3 (2014) 88-92 **Original Research**

Detection of Antibodies Against *Trypanosoma evansi* in Sheep by Indirect ELISA in Rayalaseema Region of Andhra Pradesh

S. Sivajothi[1]*, V. C. Rayulu[1], P.Malakondaiah[2], D. Sreenivasulu[3], B.Sudhakara Reddy[4]

[1]*Department of Veterinary Parasitology, College of Veterinary Science, Sri Venkateswara Veterinary University, Proddatur-516360, Andhra Pradesh, India.*
[2]*Department of Veterinary Parasitology, C.V.Sc., S.V.V.U., Tirupati, Andhra Pradesh, India.*
[3]*Associate Dean, C.V.Sc., S.V.V.U., Tirupati, Andhra Pradesh, India.*
[4]*Teaching Veterinary Clinical Complex (Veterinary Medicine), C.V.Sc., S.V.V.U., Proddatur, Andhra Pradesh, India.*

Accepted 19 Jun 2014

Abstract

The present research was carried out with an objective to improve the diagnostic tools for detection of antibodies against *Trypanosoma evansi* infection using indirect enzyme-linked immunosorbent assay (ELISA) in sheep. In this study standardized the Indirect ELISA for detection of *T. evansi* in sheep. The optimum concentration of antigen, test sera and conjugate were determined as 5µg per well, 1:10 and 1: 4000 dilutions, respectively. 464 serum samples were collected from sheep in different parts of the Rayalaseema region of Andhra Pradesh for screening of *T. evansi* infection. Out of 464 serum samples 46 (9.91%) were found positive by indirect ELISA.

Keywords: Antibodies; Diagnosis; Indirect ELISA; Sheep; *Trypanosoma evansi*

Introduction

Trypanosoma evansi is mechanically transmitted by hematophagous insects (Tabanidae and Stomoxydae) and affects many tropical regions worldwide. Natural infection is generally considered as mild or asymptomatic in sheep (Boehringer and Prosen, 1961). Parasitological examinations are usually conducted using blood, although other biological materials can be used, such as cerebrospinal fluid, joint fluid, or lymph node fluid. 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*., 2013a). 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. WBF and stained blood smear examination, the commonly used field tests for detection of *T. evansi* suffer with the least sensitivity (Sivajothi *et al*., 2013b). Microscopic observation of fresh blood is easy to carry out to detect the *T. evansi* in clinical cases. However, it is of limited sensitivity because it detects parasites when parasitaemia is above 105 trypanosomes/ml of blood. Enrichment methods are widely used, namely, Hematocrit Centrifuge Technique (Woo, 1969) or dark ground Buffy Coat Method (Murray *et al*., 1977). They increase the sensitivity of the test down to 100–200 trypanosomes/ml. If high sensitivity is required, inoculating laboratory rodents can reveal infection. It lowers the minimum level of parasitaemia detected to 20–50 parasites/ml. It provides the same range of sensitivity and specificity (90–95%) in the various host species investigated, for example, camels, cattle, buffalo, and horses (Desquesnes *et al*., 2011). 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 *Trypanosoma*

*Corresponding author: S. Sivajothi

E-mail address: sivajothi579@gmail.com

evansi infections. Meager literature was available on *T. evansi* detection by ELISA in sheep. Studies on the antibody response in sheep infected with South Indian isolate of *T. evansi* are limited; hence the present investigation was designed to detect antibodies against *Trypanosoma evansi* in sheep by indirect ELISA.

Materials and methods

Preparation of Whole cell lysate antigen of T. evansi

Trypanosoma evansi was collected from a cattle (clinically suffering with surra) presented to the College Hospital, College of Veterinary Science, Tirupati. The *T. evansi* parasites were maintained in the Wistar rats for bulk harvest of parasites. At the height of parasitaemia, the rats were bled by heart puncture and the blood sample was immediately diluted with chilled phosphate buffered saline glucose (PBS-G), pH 8.0. The separation and purification of *Trypanosoma evansi* from infected blood was accomplished by using Diethyl amino ethyl cellulose (DEAE) (S.D. Fine Chemicals Ltd, Mumbai) anion exchange column chromatography, as per method of Lanham and Godfrey (1970) with minor modifications. Height of the column, when kept 3.5 cm in a 20 ml standard glass syringe and phosphate buffered saline- glucose (5:5) were used. Whole cell lysate (WCL) antigen was prepared from purified trypanosomes described by Singh *et al*. (1994). The purified parasites were sonicated at 150W for 3-4 cycles of 30 seconds each by ultrasonic disintegrator. The sonicated material was centrifuged at 2400 x g for 20 min at 4ºC. The collected supernatant was designated as whole cell lysate antigen and stored at -20°C in 1.0 ml aliquots. The whole cell lysate antigen 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.

Collection of sera samples

Blood samples from naturally infected or suspected sheep were collected from jugular vein in separate vials without EDTA (S.D. Fine Chemicals Ltd, Mumbai) for serum collection. Serum samples were collected in sterile vials. Few drops of 1: 10,000 sodium azide solution (S.D. Fine Chemicals Ltd, Mumbai) was added. The serum samples were stored at -20°C until use.

Raising of hyper immune sera

The hyper immune sera (HIS) was raised in two healthy New Zealand white rabbits, weighing up to 1.5 to 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 an agar gel precipitation test and counter immune electrophoresis. Hyper immune sera collected from the immunized rabbits was passed through 0.45µ membrane filter, aliquoted in sterile vials (1ml/vial) to avoid multiple freeze-thaw cycle and stored at -20°C until use and it was used a 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 Enzyme linked immunosorbent assay (Indirect-ELISA)

Indirect ELISA was standardized according to Shahardar *et al*. (2008) with some modifications to detect the *T. evansi* antibodies from 464 serum samples of sheep collected during the investigation. The concentrations of the antigen, test sera and conjugate (rabbit anti bovine IgG-HRPO) for optimum performance of the assay were determined by checker board titration.

Checkerboard titration analysis

The concentrations of the antigen, test sera and conjugate for optimum performance of the test were determined by checkerboard titration. Antibody concentration was standardized by using hyper immune serum and known positive sera collected from field cases. Flat bottom, high binding capacity 96-well ELISA plates (Nunc, Denmark) were used in the study.

Sensitization of microplate wells

Microplates were coated with whole cell lysate antigen from stock at 5µg/ml of coating buffer PBS pH 7.0. It was mixed properly before dispensing 100 µl into each of 96 wells of the microplate. The sides of the plate tapped to ensure the even distribution of the antigen. The plate was sealed with a plate sealer to prevent evaporation and incubated for overnight at 4°C.

Blocking reaction

The antigen coated plates were inverted and tapped on the blotter to remove excess fluid and plates were washed 4 times with PBST (0.05 %) in the volume of 150µl/well. Then unbound sites in the wells were blocked with 1% BSA in PBST in a volume of 125 µl/well and incubated for 90 min at 25°C.

Antibody reaction

Serial log dilutions of serum of sheep were made in PBST and added in a volume of 100 µl/well. Blanks/PBST and negative serum controls were used in all the experiments. Plates were incubated for 2 hours at room temperature. Wells were washed three times in PBST with each wash of 3 min.

Reaction with antispecies antibody-enzyme-conjugate

The stock solution of donkey anti sheep IgG- ALP conjugate was separated into multiple aliquots to avoid several freezing-thawing cycles. Optimum concentration of conjugates was determined with different dilutions by checkerboard analysis. The conjugate was loaded as 100 µl/well by taking the precaution that the tip of pipette not to touch the bottom of the well. The plates were incubated for 1 hour at room temperature. Wells were washed 4 times with PBST and finally with citrate acetate buffer, pH 5.9.

Substrate / Chromogen reaction

Immediately before the end of the conjugate incubation, a working dilution of the substrate / chromogen solution was prepared. The reaction was developed by adding 100 µl substrate p-nitrophenyl phosphate at 5mg/10 ml in DEA buffer. Finally the reaction was stopped by the addition of 50 µl of 3N NaOH solution. The plate was read at 405 nm in an ELISA reader (Qualigens, India).

Interpretation of ELISA results

A cut-off value of optical density for the assay was established by the mean of the optical densities shown by the panel of negative serum samples plus three standard deviations. The sera having an optical density above the cutoff value were considered as positive. (Ethical permission was taken by the Institutional Animal Ethics Committee (IAEC) of the S.V.V.U.)

Results

In the present study, the *T. evansi* parasites isolated from a field case of cattle and were propagated in rats (Fig.1). Parasites were successfully purified by DEAE-cellulose chromatography (Fig. 2). Height of column, when kept 3.5 cm in a 20 ml standard glass syringe and phosphate buffered saline- glucose (5:5) were found the most satisfactory in performance of the present work. Concentration of WCL antigen of *T. evansi* was determined by Lowry's method as 60mg/ml. The optimum concentration of WCL Ag of *T. evansi* for coating the wells of ELISA plate determined in the study as 5µg/ml. The optimum test serum dilution found in the present study was 1:10. The optimum dilution of 1: 4000 rabbit anti-ovine Ig G-HRPO conjugate used in the study. The procedure was standardized for detection of *T. evansi* in sheep. Among 464 sheep examined in different parts of the Rayalaseema region of Andhra Pradesh for *T. evansi* infection, 46 (9.91%) were found positive by indirect ELISA.

Discussion

In the present study, the *T. evansi* parasites isolated, propagated in rats and successfully purified by DEAE-cellulose chromatography. In this procedure phosphate buffered saline- glucose (5:5) were found the most satisfactory. Singh *et al*. (1994) had also reported that the PSG (6:4) was an appropriate buffer for purification of *T. evansi*. Indirect ELISA was optimized for detection *T. evansi* antibodies in

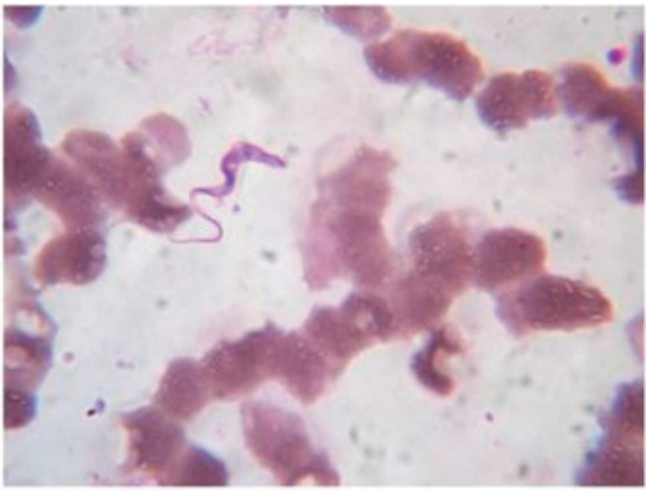

Fig. 1. *Trypanosoma evansi* parasite in leishman's stained blood smear observed under light microscope (X1000)

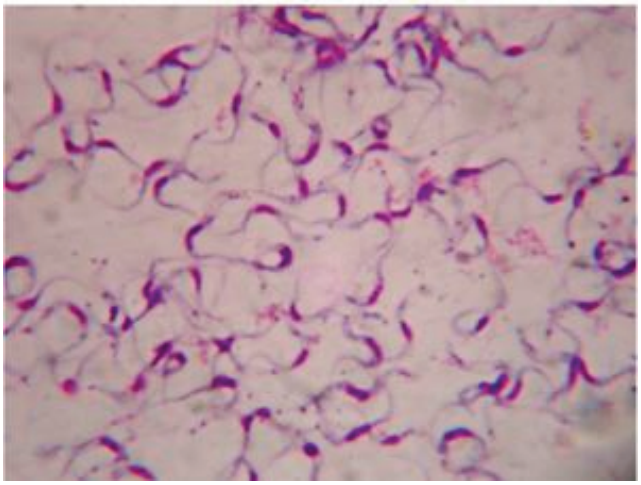

Fig. 2. *Trypanosoma evansi* parasite purified by DEAE-cellulose column chromatography and observed after leishman's staining (X1000).

sera samples collected from sheep in Rayalaseema region of Andhra Pradesh. Among the sera samples examined from 464 sheep, 46 were found positive for *T. evansi* infection. The optimum concentration of WCL Ag of *T. evansi* for coating the wells of ELISA plate determined in the study as 5µg/ml is in agreement with findings of Desquesnes *et al*. (2009) (5µg/ml). The optimum test serum dilution found in the present study was 1:10. However, little higher dilutions reported by previous workers as (1:50) by Jithendran *et al*. (1997), (1:100) by Desquesnes *et al*. (2009). The optimum dilution of 1: 4000 rabbit anti-ovine Ig G-HRPO conjugate used in the study is in agreement with Jithendran *et al*. (1997). Saseendranath *et al*. (1994) reported 83.6 per cent of sheep were positive for *T. evansi* by indirect ELISA in an experimental infection. 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.

Conclusion

In the present study, Indirect ELISA was standardized for detecting circulating antibodies of *T. evansi* in the sera of sheep. The optimum concentration of antigen, test sera and conjugate were determined as 5µg per well, 1:10 and 1: 4000 dilutions, respectively. Among 464 sheep examined in different parts of the Rayalaseema region of Andhra Pradesh for *T. evansi* infection, 46 were found positive by indirect ELISA.

Acknowledgements

The authors acknowledge the authorities of Sri Venkateswara Veterinary University, Tirupati for providing facilities to carry out this research. Authors are also thankful to the field veterinarians who assisted in the collection of blood samples.

References

Boehringer, E.G., Prosen, A. F., 1961. Transmision experimental del Mal de Caderas. Anales del Instituto de Medicina Regional Argentina Vol. 5, p. 69.

Desquesnes, M., Bossard, G., Thévenon, S., 2009. Development and application of an antibody-ELISA to follow up a *Trypanosoma evansi* outbreak in a dromedary camel herd in France. Veterinary Parasitology 162(3-4), 214–220.

Desquesnes, M., Kamyingkird, K., Vergne, T., Sarataphan, N., Pranee, R., Jittapalapong, S. 2011. An evaluation of melarsomine hydrochloride efficacy for parasitological cure in experimental infection of dairy cattle with *Trypanosoma evansi* in Thailand. Parasitology 138(9), 1134–1142.

Desquesnes, M., Bossard, G., Thevenon, S., Patrel, O., Lepetitcolin, E., Hollzmuller, P., Berthier, D., Jacquiet, P., Cuny, G., 2009. Development and application of an antibody-ELISA to follow up a *Trypanosoma evansi* outbreak in a dromedary camel herd in France. Veterinary Parasitology 162, 214-220.

Jithendran, K.P., Rao, J.R., Mishra, A.K., 1997. Evaluation of antigenic preparations for the diagnosis of experimental *Trypanosoma evansi* infection in bovine calves. Journal of Veterinary Parasitology 11(1), 17-21.

Lanham, S.M., Godfrey, G.D., 1970. Isolation of salivarian trypanosomes from man and other animals using DEAE- cellulose. Experimental Parasitology 28, 521-534.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R., 1951. Protein estimation with the folin-phenol reagent. Journal of Biological Chemistry 193, 265-275.

Murray, M., Murray, P.K., McIntyre, W.I.M., 1977. An improved parasitological technique for the diagnosis of African trypanosomiasis. Transactions of the Royal Society of Tropical Medicine and Hygiene 71(4), 325–326.

Saseendranath, M.R., Ramkrishna, J., Ramdass, P., Gunaseelan, L., 1994. Antibody detection enzyme linked immunosorbent assay(Ab-ELISA) in the diagnosis of *Trypanosoma evansi* infection in sheep. Journal of Veterinary and Animal Sciences 25, 123-126.

Shahardar, R.A., Rao, J.R., Mishra, A. K., Tewari, A. K., 2008. Detection of antibodies against *Trypanosoma evansi* in dromedary camels by ELISA using purified antigens. Journal of Veterinary Parasitology 22(2), 47-51.

Singh, V., Chhabra, M.B., 1993. Counter immune-electrophoresis for rapid detection of circulating antigens of *Trypanosoma evansi*. Indian Journal of Animal Sciences 63 (6), 625-627.

Singh, V., Singh, A., Chhabra, M.B., 1994. Polypeptide profile of whole cell lysate of *Trypanosoma evansi* stocks from northern India. Indian Journal of Animal Sciences 64, 14-17.

Sivajothi, S., Rayulu, V.C., Sudhakara Reddy, B., 2013a. Haematological and biochemical changes in experimental *Trypanosoma evansi* infection in rabbits. Journal of Parasitic Diseases DOI 10.1007/s12639-013-0321-6

Sivajothi, S., Rayulu, V.C., Sujatha, K., Sudhakara Reddy, B., Amaravathi, P., 2013b. Histopathological Observations in Rabbits Experimentally Infected with *Trypanosoma evansi*, Journal of Advanced Veterinary Research 3, 122-126.

Woo, P.T.K., 1969. The haematocrit centrifuge for the detection of trypanosomes in blood. Canadian Journal of Zoology 47(5), 921–923.