3.0 MATERIAL AND METHODS

3.1 General conditions

3.1.1 Sterilization of glassware and plastic ware
During the course of this study Borosil or Corning brand of glassware and plastic ware (M/s. Tarsons Ltd.) were used. All the glassware were cleaned and sterilised as per standard procedures. Plastic wares were also prepared as per standard procedure.

3.1.2 Laboratory chemicals
The laboratory reagents and buffers used in this study were prepared in double glass distilled water using ‘Excellar’ or guaranteed reagent quality chemicals. The other chemicals viz., rabbit anti-bovine IgG conjugate, SDS-PAGE standard molecular weight protein marker were procured from Bangalore Genei Co., Bangalore. The reagents viz., ortho-phenylene di hydrochloride (OPD) and ortho-dianisidine dihydrochloride (ODD) were obtained from Sigma Company (USA). The ELISA plates (Titertex) were procured from Flow Laboratories (Netherlands).

3.2 Experimental animals

3.2.1 Rats
Albino rats of either sex weighing about 150 to 200 gm were procured from Small Animal House, Dept. of Livestock Production and Management, Veterinary College, Hebbal, Bangalore, for routine maintenance of Trypanosoma evansi isolate in the laboratory and for collection of blood for preparation of antigen.

3.2.2 Rabbits
New Zealand White rabbits were also obtained from Small Animal House, Dept. of Livestock Production and Management, Veterinary College, Hebbal, Bangalore, for raising hyperimmune serum.
3.3 Ethical considerations

Prior permission from Institutional Animal Ethics Committee (IAEC), Veterinary College, Hebbal, Bangalore was obtained for the use of laboratory animals in the present study.

3.3.1 Isolate of *T. evansi*

*Trypanosoma evansi* isolate was obtained from a clinical case of Surra in buffalo from East Godavari district of Andhra Pradesh state. The infected blood was inoculated intraperitoneally into the rats at the rate of 0.5 ml and brought to the laboratory and maintained.

3.3.2. Maintenance of Trypanosoma isolates

The parasites were maintained in rats by inoculation method based on the infectivity titration (Lumsden *et al*., 1973). Convenient passage dose and passage interval for rats was arrived and this method essentially consisted of examination of wet blood film from the peripheral blood of rats and scoring the degree of parasitaemia as follows.

R → less then one trypanosome per microscopic field  
+ → 1-5 trypanosomes per field  
++ → 6-10 trypanosomes per field  
+++ → 11-20 trypanosomes per field  
++++ → Teeming parasites

The working dose for routine passage was arrived at by dilution of *T. evansi* infected blood through tail bleeding in Alsever’s solution at the +++ level of parasitemia, so as to obtain an inoculation of $10^4$ to $10^5$ organisms for intra-peritoneal inoculation to uninfected rats. Thereafter, the parasitaemia was routinely monitored for further passage and the passage interval was five to six days in rats.
3.3.2.1 Counting of trypanosomes

The trypanosomes were counted by the method of Janeen et al. (1972). Two ml of WBC diluting fluid was taken in a watch glass and one drop of one per cent aqueous solution of gentian violet was added to it. The diluted blood was drawn up to 0.5 mark in a WBC pipette followed by diluting fluid up to mark 11. It was mixed gently and the neubaur counting chamber was charged with a drop of material. After one to two minutes the total number of trypanosomes per microlitre of the diluted blood were counted, multiplied by 1000 and the trypanosomes per ml of diluted blood were calculated.

3.3.2.2 Collection of blood for Trypanosoma evansi isolation

Following the administration of infective inoculation of T. evansi to rats, the course of infection was monitored. At the height of parasitaemia the rats were mildly anaesthetised using chloroform and bled by heart puncture using heparinised (10-20 IU of Na salt of heparin per ml blood) glass syringe. The blood samples were then immediately diluted 1:3 with chilled phosphate saline glucose (PSG) buffer (P$^+$H 8.0) and processed for chromatographic purification of trypanosomes.

3.3.3 Separation of Trypanosomes

The trypanosomes were separated from the experimentally infected blood of rat through Di Ethyl Amino Ethyl (DEAE) cellulose columns as per the method of Lanham and Godfrey (1970) and Srivastava et al. (1988). The DEAE-cellulose slurry was prepared by swelling of the cellulose powder in N/10 NaOH (1g/10 ml) for one hour with intermittent stirring. The cellulose was then washed with distilled water, three to four times by decantation. The pH was brought to neutral using orthophosphoric acid. The sediment was then mixed with N/10 HCl (1 g/10 ml) and allowed to settle down for an hour and washed with distilled water for three to four times. The slurry was equilibrated with phosphate saline glucose (PSG) buffer, pH 8.0 by repeated washings.

A simplified method of trypanosome purification by mini-anion exchange chromatography was used to fractionate the trypanosomes as per method of Srinivas, (2000). A glass syringe without plunger fitted with a hypodermic needle (20G) and into which a filter paper disc was placed at the bottom was used as a working column. About 2.0 to 2.5 ml slurry was loaded into the column and equilibrated to pH 8.0 with repeated flow of PSG buffer. Then the sample of blood collected in heparin and diluted 1:3 in
chilled PSG as previously described, was loaded onto the DEAE slurry slowly through the sides of the column with the help of pasture pipette. After the blood sample entered the bed, small quantity of PSG was applied above the blood in the column. The column outlet was opened till a few milliliters of column elute was obtained and it was then centrifuged at 3000 rpm for 10 min and the residue was examined for red blood cells (RBC). After confirming that the column elutes were free from RBC, the outlet of the column was opened for chromatographic separation of trypanosomes and the column elutes were examined to spot the fractionated trypanosomes under microscope. The elute was collected in a glass beaker on ice. The elute thus collected was centrifuged at 4°C for 10 min at 3000 rpm. The sedimented trypanosomes were resuspended in chilled PSG buffer and washed by centrifugation three times for ensuring pure suspension of parasites free from blood components.

3.4 preparation of whole cell lysate antigen of T.evansi

The whole cell lysate T. evansi, antigen was prepared from DEAE-cellulose chromatography fractioned trypanosome population. The pelleted trypanosomes were mixed with six to eight ml of PBS, pH 7.4 and subjected to ultra sonicication at 14 hz for 30 sec. (Soneprep MSE-150) (peak to peak) for three times with an interval of one minute each in the presence of phenyl methyl sulphoxynyl fluoride (PMSF) (10 µg/ml). The resulting suspension was centrifuged at 12000 rpm at 4°C for 20 minutes. The supernatant was kept in aliquots at −20°C and designated as whole cell lysate / soluble antigen.

3.4.1 Protein estimation

The protein concentration of the whole cell lysate antigen of T. evansi was estimated by Brad ford method (Brad ford, 1976)

3.4.2 Epidemiological studies

3.4.2.1 Study area

The study area included three districts of Andhra Pradesh viz., East Godavari, Krishna and Guntur and three districts viz., Davanagere, Chitradurga and Shimoga of
Karnataka state. The districts selected in Andhra Pradesh comes under Krishna-Godavari Zone whereas Chitradurga and Davanagere falls under central dry Zone and Shimoga categorized as trans south Zone of Karnataka. Particulars of animals like age, sex, history and clinical manifestations exhibited were documented during the study period during different seasons (Table 1) since endemic reports on prevalence of T.evansi were made by earlier workers.

3.4.3 Diagnosis of Trypanosoma evansi infection in Bovines

The animals were screened for infection by different methods such as wet blood film examination, Giemsa’s staining, Buffy Coat Technique, Quantitative Buffy Coat including Indirect Enzyme Linked Immuno Sorbent Assay and Enzyme Immuno Transfer Blot.

3.5 Wet blood film examination

A small drop of fresh blood was placed on a clean microscopic glass slide and covered with a clean cover slip to spread the blood as a monolayer of cells and examined under the low power of the compound microscope for the presence of active movement of trypanosomes (Veer singh and Yogesh singh,2005)

3.5.1 Giemsa staining method

Blood smears were fixed with methanol for 30 seconds and allowed to dry. Stain in a 1 in 20 dilution of Giemsa stain for 20 min and washed with distilled water. Poured off the stain and flushed by pouring of distilled water. Allowed the slide to dry and examined under compound microscope in oil immersion(Veersingh and Yogesh singh,2005).

3.5.2 Buffy coat technique (BCT) for the detection of T. evansi The blood was collected into the vials containing EDTA (1mg/ml) from cattle and buffaloes. The blood was filled into the Wintrobe test tube up to the middle and centrifuged at the rate of 3000 rpm for 10 mins. After centrifugation, the plasma (top most layer) was removed carefully with a help of hypodermic needle fitted to a syringe and discarded. Then the buffy coat layer was extracted cautiously and expelled onto a clean glass slide and examined for T.evansi organisms for their motility. After observing the organisms, the buffy coat was
allowed to dry, fixed in methanol and stained by Giemsa method. This procedure was as per Lynneshore Garcia, (2001).

3.5.3 Quantitative Buffy Coat (QBC) method for detection of

*T.evansi* organisms

The detection of the *T. evansi* organisms in the experimentally infected mice was made by the QBC method (Levine *et al* 1989). In this method, the blood from the tail of the mice was collected directly into the QBC-V tube (acridine coated) carefully without air bubbles and rotated gently with the thumb and index fingers towards the acridine coated end of the tube. The end was then closed with a plastic closure and to the opposite end a plastic float was inserted. Then the QBC-V tubes were placed in the QBC centrifuge (Beckton Dickinson CO.), and were centrifuged for five minutes with 12000 rpm. Then the immersion oil was applied over the buffy coat area (examined under the paralens) by placing the QBC-V tube in a paraviewer using the flurosence microscope for the detection of the *T. evansi* organisms. In this technique haemo parasites are concentrated in to a small buffy coat region and are held close to the wall of the tube by the plastic float, thereby making them readily visible by microscope. The tubes pre-coated with acridine orange provide a stain which induces metachromatic flurescene in haemoparasites.

3.5.4 Collection of test serum samples

Five ml of blood was collected from bovines in sterilised and clean vials and serum was collected and stored as aliquots at −20°C till further use.

A total of 25 serum samples were collected from cattle and buffaloes, which were found positive for *T.evansi* organisms. Similarly, 25 serum samples were collected from animals which were not found infected and also from a farm in Bangalore with no history of Trypanosomosis. No endemic focus has been described from animals in and around Bangalore.

3.5.5 Raising of hyper immune serum (HIS)

Hyper immune serum was raised in the healthy rabbits as per Dhami (1996). The antigen (500µg protein) was injected subcutaneously after mixing with equal quantity of Freund’s complete adjuvant (FCA). The second injection was given subcutaneously after one week interval by mixing the antigen with equal quantity of Incomplete Freund’s adjuvant (IFA). The remaining three injections of antigen with equal quantity of Incomplete Freund’s adjuvant (IFA) were given intramuscularly at weekly intervals. Then the rabbits were bled seven days after the last booster and the serum was separated, stored at −20°C and checked for its specificity by counter immuno electrophoresis.

3.5.6 Counter Immunoelectrophoresis (CIEP) for checking the

hyperimmune serum
Counter immuno electrophoresis was performed as per Veer Singh and Chhabra (1993) with some modifications. One per cent agarose was prepared in Tris borate buffer with pH 8.2 to 8.4 by adding 0.25 g of agarose in 25 ml of Tris borate buffer (Appendix). Onto a clean glass slides five ml of the buffer gel was poured and allowed to solidify. Two wells of four mm diameter were cut with a well distance of six mm. The well to be located on the cathode side was filled 10µl with *T. evansi* antigen and the well at the anodic side with hyper immune sera. Electrophoresis was carried out in a suitable trough containing Tris borate buffer. The slides were placed on the rack and each end was connected with a filter paper wick dipped in buffer. A current of 100 mA per slide was applied for 90 minutes.

The slides were washed in normal saline and stained with Coomassie brilliant blue for five minutes and destaining was done till the bands could be visualised.

3.6. Sodium Do-decyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out as per description of Laemmli (1970) using discontinuous gel and buffer system using minidual slab gel electrophoresis (Bangalore Genei) system. Two glass plates (one with notch and one without notch), two spacers and comb were washed thoroughly, rinsed with 70 per cent alcohol and dried. Bottom of the glass plates were sealed with one per cent agarose. Separating gel (10%) was prepared and poured between plates with a gentle continuous flow avoiding air bubbles up to about 3/4th height of the plate. The gel was overlaid with water saturated N-butanol immediately to avoid air contact and the gel was allowed to set at room temperature for 45 minutes. After polymerisation the overlay was poured off and separating gel was washed with double distilled water and subsequently dried with the blotting paper. Stacking gel was prepared and poured up to the required height. The comb was placed and the gel was allowed to set for 10 minutes. The comb was removed and the slab was fixed to the electrophoresis apparatus. Running buffer was poured into both upper and lower buffer tank.

3.6.1 Preparation of sample and protein marker
The required protein concentration of *T. evansi* (50 μl) was taken with 12.5 μl of sample buffer and 5 μl of tracing dye. Similarly protein markers obtained from Bangalore Genei of molecular range 14.3 to 97.4 kDa was prepared for carrying out electrophoresis. The sample and protein markers were boiled in water bath at 100°C for five minutes.

The denatured samples were loaded in separate wells and electrophoresis was carried out at a constant current of 10 mA (50v) for stacking gel and 16 mA (100v) for the resolving gel using power pack (Bangalore Genei). When the dye front reached the bottom of the gel, the power pack was switched off. Gel was removed and stained with Coomassie blue stain.

### 3.6.2 Coomassie Brilliant blue staining procedure

Coomassie Brilliant blue staining was carried out according to the description of Laemmli (1970). After completion of electrophoresis slowly the spacers and either of the plate was removed. The gel was immediately and carefully transferred to the staining solution. The gel was allowed in the staining solution overnight at room temperature. Then the gel was transferred to destaining solution. The destaining solution was changed every 20 minutes till the bands were clearly seen.

### 3.6.3 Determination of molecular weights of peptide

The relative mobility of each peptide was calculated using the following formula:

\[
R_f = \frac{\text{Distance migrated by the polypeptide}}{\text{Distance migrated by tracking dye}}
\]

A standard curve of log M<sub>r</sub> of the markers versus Rf values was plotted and the regression equation determined. Molecular weight of poly peptides of whole cell lysate antigen of *T.evansi* was calculated using the regression equation with semi log graph sheet.
3.7. Indirect Enzyme Linked Immuno Sorbent Assay

The Indirect ELISA was carried out to detect anti-\textit{T. evansi} antibodies as per the method of Baghel \textit{et al.} (1995) with minor modifications.

\textbf{Antigen}: \textit{T. evansi} soluble antigen was prepared as described earlier.

\textbf{Serum samples}: Sera samples from bovines collected from three different districts each of Andhra Pradesh and Karnataka were screened by ELISA.

\textbf{Buffer, conjugate and equipment}: Carbonate buffer pH 9.6 was used to coat antigen in 96 well flat bottom polystyrene plates (Titertek). Rabbit anti-bovine horse radish peroxidase (HRPO) used as conjugate and O-phenylene diamine (OPD) in phosphate-citrate buffer of pH 5.0 with hydrogen peroxide (30\% v/v) was used as substrate solution. Readings were taken in multiscan plus P (Lab Systems) ELISA reader at 492 nm.

3.7.1 Determination of optimal sensitizing dose of antigen: Optimal concentration to be coated for antigen was determined by checker board titration with known positive serum samples for \textit{T evansi} antibodies. The lowest concentration of antigen which showed the maximum ELISA titer was chosen as optimum sensitizing dose.

Titertek polystyrene plates were coated with \textit{T. evansi} antigen containing five \(\mu\)g/well in carbonate buffer (pH 9.6) and kept at 4\(^{0}\)C for overnight. The wells were emptied and washed thrice with washing buffer (PBS-T) and 100 \(\mu\)l of blocking buffer was added to each well and kept at 37\(^{0}\)C for one and half hours. After blocking, the wells were emptied and washed with PBS-T thrice. Then 100 \(\mu\)l of diluted sera was added to the wells in duplicate. To the last two wells, a known positive serum and new born calf serum as known negative serum was added and plates were incubated at 37\(^{0}\)C for two hours. Subsequently, plates were washed three times with PBS-T and dried thoroughly and 1:1000 dilution of rabbit anti-bovine IgGHRPO conjugate in blocking buffer at the rate of 100\(\mu\)l per well was added, mixed gently and kept at 37\(^{0}\)C for one and half hours. The plates washed with PBS-T three times and tapped dry. Freshly prepared OPD substrate (5 g/12.5 ml) in citric acid phosphate buffer and 0.05 per cent hydrogen peroxide (50 \(\mu\)l/well) was added, mixed gently and kept in dark for 10 minutes and
colour development was monitored. The reaction was arrested with 50 µl of 1N sulphuric acid and readings were taken in multiscan plus P (Lab systems) ELISA reader at 492 nm. The OD value of any serum sample greater than the cut off value was regarded as positive.

3.7.2 Determination of cut off value : To determine the cutoff value newborn calf serum samples were used. The cutoff value was calculated by taking mean absorbance values of known negative sera plus three standard deviation. The serum sample with OD values above the cut off value was regarded as positive.

3.7.3 Enzyme Immuno Transfer Blot (EITB)

The EITB was carried out as per the method of Towbin et al. (1979). SDS-PAGE was carried out first as previously described using 10 per cent resolving gel. *T. evansi* soluble antigen containing 40 µl, 50 µl and 60 µl of protein were loaded to each lane along with protein markers in the range of 14.3 to 97.4 kDa. Both antigen and protein marker was prepared as described earlier. After electrophoresis, the marker lane was cut and stained with Coomassie blue staining solution. The remaining gel was used for electro transfer blot.

Before carrying out the transfer step, the gel was washed thrice with transfer buffer with a gap of five minutes. Nitrocellulose membrane (0.45 µm) and blotting paper of size 8 cm x 7.5 cm was cut and immersed in transfer buffer for 30 min. One sheet of blotting paper was transferred onto the electroblot stage and rolled over the blotting paper to remove the excess water or air bubbles. Similarly, another two sheets were placed then followed by nitrocellulose membrane and gel. Again three layers of blotting paper was placed and trapped air was removed. Transfer was performed with trans-blot semidry electrophoresis transfer cell (BioRad) for 45 min at a constant voltage (12v).

Once the transfer was completed the membrane was immersed in blocking buffer overnight at 4°C. The membrane was washed three times at the rate of five minutes each time. then the positive serum was added (250 µl to 1 ml of blocking buffer) to the nitrocellulose membrane and kept for one hour at room temperature. Then it was washed three times with washing buffer for two to three minutes each time and rabbit anti-bovine
IgG HRP conjugate was added to the NCM and kept undisturbed for one hour at room temperature (10 ml of blocking buffer with 10µl of conjugate). Rabbit anti-bovine IgG HRP conjugate was prepared by mixing 100µl of conjugate in 400µl of distilled water. Then it was washed with the washing buffer and then freshly prepared ODD solution was added to the NCM and kept for 10 to 15 minutes with occasional shaking for the presence of bands on the NCM. The bands were compared with the standard molecular weight protein marker to know the immuno reactive peptide.

3.8 Sensitivity and specificity of EITB and ELISA

The sensitivity and specificity of EITB and ELISA was calculated by the following formula

\[
\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive} + \text{false negative}} \times 100
\]

\[
\text{Specificity} = \frac{\text{True negative}}{\text{True negative} + \text{false positive}} \times 100
\]

True positive was positive sample by Giemsa staining as well as by wet blood film examination. False positive means was positive in wet blood film examination and negative in Giemsa staining. True negative implied negative by wet blood film and Giemsa staining where as false negative indicated positive by Giemsa staining and negative by wet blood film examination.

3.9 Statistical analysis

The data obtained were analysed by chi-square test using the software Graph pad prism.