circulating in rural areas. The strains were typed as isoenzyme variants with *T. cruzi* as the principal zymodeme ZI (*T. cruzi* I). They concluded an effective program of epidemiological vigilance is required to prevent the spread of *T. cruzi* I strains into human dwellings.

Njiru *et al.* (2007) investigated the use of inter-simple sequence repeats and microsatellites in revealing polymorphism among *T.evansi* isolates.

### III MATERIAL AND METHODS

#### 3.1 General Conditions

**3.1.1 Sterilization of glassware and plasteware**

During the course of this study Corning or Borosil brand of glasswares’ and plastic ware (M/s Tarsons Ltd.) were used. All the glasswares’ were cleaned and sterilized as per standard procedures. Plasteware were also sterilized as per standard procedures.

**3.1.2 Laboratory chemicals**

The laboratory reagents and buffers used in this study were prepared in double glass distilled water using Excelar reagent quality chemicals. The other chemicals included EDTA, heparin, Giemsa’s stain, methanol, D-glucose, citric acid sodium citrate, disodium hydrogen phosphate, sodium di hydrogen phosphate, sodium chloride, potassium chloride, bovine albumin fraction-V(HiMedia lab ltd, Mumbai), phenyl methyl sulphonyl fluoride, glycerol, chloroform, DEAE-cellulose (Whatman DE 52), orthophosphoric acid, Bradford protein estimation Kit, (Bangalore Genei Co., Bangalore), glutaraldehyde, 100 bp DNA marker (Bangalore Genei Co., Bangalore), primers (Bioserve, Biotechnologies (India) Pvt Ltd, Hyderabad), PCR master mix (Genetix Biotech Asia Pvt Ltd, New Delhi) , Taq Polymerase (Genetix Biotech Asia Pvt Ltd, New Delhi), TAE (50X) [Genetix Biotech Asia Pvt Ltd, New Delhi], absolute ethanol (E-Merck, Germany), ethidium bromide (10mg/ml) [Genetix Biotech Asia Pvt Ltd, New Delhi, Agarose (Sigma-Aldrich Chemical Pvt Ltd), Nuclease –free water, Frozen blood genomic DNA spin- 100 Chromous Bio Tech PVT. Ltd, Bangalore.

#### 3.1.3 Materials and Equipments
Micro centrifuge (Mikro-120, Hettich), Spinix, Thermostat plus (Eppendorf, Germany), Agarose gel electrophoresis system with power supply (Bangalore Genei/Max well, Germany), Micro-wave oven (LG, India), Gel documentation system (Bio-Rad, Germany), Thermal cycler (Eppendorf, Germany), Mini cooler, Incubator (Sanyo Electric CO. Ltd, Japan), Sonicator (Soniprep 150, MSE), Micropipettes (Microlitf), ‘U’ shaped microtitre plate (Laxbro Manufacturing Co. Pune), Glass Syringe 20 ml, Hypodermic syringe, Disposable syringes of 5 and 10 ml, Whatman filter paper No 41 were used in the study.

3.1.4 Experimental Animals

The experimental animals were maintained in the tick and fly proof shed of the Department of Parasitology using standard feeding regimes. The permission for their use of was obtained as per No.LPM/IAEC/2008 dt1/3/2008 from the Chairperson of IAEC/CPCSEA, Professor and Head, Dept of LPM, Veterinary College, Bangalore.

3.1.4.1 Mice

Albino mice of either sex, 6-12 weeks of age weighing 25-35g were procured from the Laboratory Animal House, Veterinary College, and Bangalore. These animals were used for routine laboratory passage of the isolates of Trypanosoma evansi.

The mice were carried to the blood collection spot and 0.75 ml of pooled blood samples with Alsever’s solution from wild animal species were inoculated to each mouse.

3.1.4.2 Rats

Wistar rats of either sex weighing 200-300g were procured from the Laboratory Animal House, Veterinary College, Bangalore, and used for bulk harvests of parasites for trypanosomal antigen preparations.

3.1.5 Study area

The molecular and serological studies on trypanosomosis in captive wild animals were conducted with the blood samples collected from the Bannerghatta Biological Park (Bangalore), Shri Jayachamrajendra
Zoological Park at Mysore, and Tiger-lion safari at Thyavarekoppa and elephant camp at Sakkarebyalu (Shimoga district). The blood samples were collected from a camel herd at Hebbal, Bangalore.

3.1.6 Collection of Material

The blood samples were collected from captive wild animals viz, tigers, lions, leopards, jaguars and elephants. Blood samples were also collected from camels which were from Rajasthan State. The blood sample was drawn from the coccygeal vein from the tigers, lions, leopards and jaguars restrained in the squeeze cage. The blood samples of elephants were collected from marginal ear vein. The jugular or mammary veins were used for collection of blood from camels. About 4.5 to 5 ml of blood was drawn in 5ml sterilized hypodermic syringe.

3.1.6.1 Blood smear

Blood smears were prepared at the spot of collection and were labeled. They were air dried, fixed with methanol and were stained with Giemsa’s stain (1:4 dilution).

3.1.6.2 Collection of serum samples

One hundred and fifteen blood samples were collected. Out of the 5ml of blood collected 2.5 ml of blood was poured into sterile glass test tubes for serodiagnosis. Serum was separated and centrifuged at 4000 rpm for 5 minutes in a refrigerated centrifuge (4°C). It was then stored in small aliquots at -20°C till further use. The serum was decomplexed by incubating at 56°C for 30 minutes for the Passive haemagglutination test.

3.1.6.3 Collection of blood sample for PCR

About 1.5 ml of collected blood was poured into cryovials containing EDTA and stored at -80°C till further use.

3.1.6.4 Mouse inoculation for isolation of isolates.
3.1.6.4.1 Isolates from wild animals

Immediately after collection of blood from the study animals about 0.2 ml of each blood sample was taken into a vial containing Alsever’s solution and 0.3 ml of the blood sample was inoculated to mice by intra peritoneal route.

3.1.6.4.2 Isolation of canine isolates.

*Trypanosoma evansi* dog isolate was obtained from Khanapur in Belgaum district and the trypanosome antigen was prepared.

3.1.7 Quantitative Buffy coat (QBC) method for detection of *T.evansi* organisms

The detection of the *T.evansi* organisms in the experimentally infected mice was carried out by the QBC method (Levine *et al*. 1989). 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 cork 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 at 12000 rpm. Then cedar wood oil was applied over the buffy coat area for examination under the paralens attached to the compound microscope by placing the QBC-V tube in a paraviewer for the detection of the *T.evansi* organisms. In this technique haemoparasites are concentrated into 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 fluorescence in haemoparasites.

3.1.8 Parasite isolate and its Maintenance.

3.1.8.1 Maintenance of *Trypanosoma evansi* isolate

The parasite isolate was maintained in the laboratory by animal inoculation method. Based on the infectivity titration for *T.evansi* (Lumsden *et al*. 1973), an optimal passage dose and optimal passage interval for mice and rats was arrived. The method essentially consisted of examination of wet blood films from the peripheral blood of laboratory animals under x 400 magnification and scoring the degree of parasitaemia. The scoring of the degree of parasitaemia was as follows:
0 No parasite detected.

+ 1-5 parasites detected per microscopic field.

++ 6-10 parasites detected per microscopic field.

+++ 11-20 parasites detected per microscopic field.

++++ More than 20 trypanosomes per field.

M Massive infection i.e. organisms in teeming number of trypanosomes are equal or higher than the number of erythrocytes per fields.

The working dose for routine passage in rodents was arrived at by dilution of *T.evansi* infected blood through tail bleeds in Alsever’s solution pH 8.0 at +++ level of parasitaemia, so as to obtain an inoculum of $10^4$ to $10^5$ organisms for intraperitoneal inoculation of mice and rats. The passage interval for mice and rats was 3-4 days and 5-6 days, respectively.

### 3.1.8.2 Preparation of stabilate

Cryopreservation was used for preservation of parasite isolates in the laboratory and the cryostablates were prepared as described below.

*Trypanosoma evansi* infected blood was collected from rats, as well as mice at the height of parasitaemia. The animals were anaesthetized by mild chloroform anaesthesia and bled by heart puncture in heparinised 5.0 ml glass syringe using a 22 G hypodermic needle. The freshly collected blood was carefully mixed with glycerol (10% of total volume of blood collected). Stabilates were set up in cryo capillary tubes and were placed in cryovials. These cryovials were secured in a canister. The canisters were placed at -70°C overnight, preceding a brief exposure at -20°C for 30 min. The material was finally transferred to the vapour phase of liquid nitrogen.
3.1.9 Serological test – Passive Haemagglutination (PHA)

3.1.9.1 Preparation of Antigen

_Trypanosoma evansi_ canine isolate maintained in albino mice served as source of antigen.

3.1.9.1.1 Isolation of host cell-free trypanosomes.

Host cell-free trypanosomes were obtained by DEAE-cellulose chromatography of infected blood following the method of Lanham and Godfrey (1970) with minor modification.

3.1.9.1.1.1 Equilibration of DEAE-cellulose

A total quantity of 12.5 g DEAE-cellulose (Whatman DE 52) was suspended in 50 ml of phosphate saline glucose (PSG) buffer using the range of 6:4 (4 parts distilled water). The pH of a PSG suspension was lowered to pH 8.0 with 5% H₃PO₄. The main bulk of the exchanger was allowed to settle for 90 minutes and the supernatant fluid was removed by decantation. The DEAE-cellulose was washed further four times by decantation with 50ml buffer each time and the suspension was poured on to the filter paper placed in a funnel and washed with 200 ml buffer. The buffer (50 ml) was added to the slurry, mixed well and was used. When the slurry was used within 24 hours it was left at 4°C and thawed at room temperature before loading to the column.

3.1.9.1.1.2 Column preparation

A sterile 20 ml glass syringe without plunger was fitted with a hypodermic needle (18 G) and used as working column. A Whatman No 41 filter paper disc having same diameter of syringe was placed inside the syringe. The syringe was carefully placed to the burette stand. PSG buffer was poured into the syringe so that the filter paper disc settled to the floor. The equilibrated slurry was stirred thoroughly with glass rod so that it formed a uniform suspension with buffer. The slurry was loaded into the column from the sides with the help of a dropper or Pasteur pipette. Once the slurry started settling down there was good out flow of buffer drop by drop from the hypodermic needle. The slurry was added till the column length was 8 cm. Once the slurry settled, it was washed with 200 ml of buffer, by pouring slowly from the sides without
disturbing it. Caution was taken so that few centimeters of buffer layer was always maintained, so that the slurry did not dry up. When the column was not in use the flow of elute was stopped by plugging the syringe outlet with parafilm and then a needle was placed back, so that the flow of elute was completely stopped.

3.1.9.1.1.3 Collection of blood for isolation of trypanosomes

The inoculum of *T. evansi* was administered to rats and the course of the infection was monitored from tail bleeds. At the peak parasitaemia, the rats were anaesthetized using chloroform and bled by heart puncture using heparinized syringe (10-20 I.U). The blood samples were then immediately diluted 1:3 with chilled phosphate buffered saline glucose (PBS-G), pH 8.0 and processed for chromatographic purification of the trypanosomes.

3.1.9.1.1.4 Separation of host cell-free trypanosomes

The buffered blood was subjected to centrifugation at 2000 rpm for 4 minutes so that the RBC’s did not settle completely. The plasma formed a cloud like appearance on the partially settled RBC’s and with a pipette the plasma layer was separated. It contained sufficient number of RBC’s and other cells along with large number of trypanosomes.

PBS-G was again added to the RBC sediment and was mixed gently. It was again subjected at slow centrifugation speed and the procedure repeated as described above. This step was repeated for 5 to 6 times till the RBC sediment showed few organisms.

This chromatography was performed at room temperature and the purified trypanosomes were then pelleted by centrifugation at 1500 g for 10 minutes. The pelleted trypanosomes were resuspended in fresh PBS (pH 7.4) for further use.

3.1.9.1.1.5 Sonication

The purified Trypanosome pellet thus collected was mixed with phosphate buffer saline of pH 7.4 and sonicated in the presence of phenyl methyl sulphonyl fluoride (PMSF) (1µg/ml) at peak to peak amplitude for 30 seconds three times at 4°C with an interval of one minute each in an ultra sonicator. The sonicated suspension was centrifuged at 10,000 g for 15 minutes at 4°C., the supernatent was collected and designated as soluble antigen and was stored at -20°C for further use.
3.1.9.1.1.6 Protein estimation

The protein concentration of the soluble trypanosome antigen was estimated as per the method of Bradford (1976) using protein estimation kit from Bangalore Genei Co., Bangalore.

3.1.9.1.1.7 Sensitization of sheep RBCs

Sensitization of sheep erythrocytes with soluble antigen was carried out as per the procedure of Jatkar and Singh (1971). Ten ml of sheep blood was collected aseptically in equal volume of Alsever’s solution and allowed to stand for 24 hours at 4°C. It was washed three times in phosphate buffer saline albumin (PBSA) at 500 g for 15 minutes and finally 2.5% sheep erythrocytes suspension was prepared in PBSA.

The above erythrocyte suspension was treated with 2.5% glutaraldehyde by adding drop by drop (8ml of 2.5% sheep erythrocyte suspension with 1ml of 2.5% glutaraldehyde) stirred gently at 37°C for one hour and then washed thrice. The glutaraldehyde fixed erythrocyte suspension was stored at 4°C for further use.

Three ml of PBSA was mixed with one ml of diluted antigen (1/20 concentration) and to this 1 ml of 2.5% glutaraldehyde fixed sheep erythrocyte suspension was added. This was mixed thoroughly at 37°C for 30 minutes and washed three times at 500 g cycles for 10 minutes.

The above packed antigen coated cells were suspended in 5 ml PBSA. Optimum sensitizing concentration of antigen was achieved at 1:20 dilution of antigen containing 1mg of protein per 1ml.

3.1.9.1.1.8 PHA test proper

PHA test was carried out as described by Saseendranath et al. (1995) in which 25µl of phosphate buffer saline (PBS) of pH 7.4 was dispensed to each well of U shaped microtitre plate. Serial two fold dilution of the test serum in PBS was made starting from 1:2 up to 1: 4096 dilutions. Equal volume (25µl) of sensitized erythrocyte suspension was added to each well and incubated at 37°C for one hour. The known positive and negative serum samples of leopards from naturally infected leopards of Thyavarekoppa Lion and Tiger Safari and three month old cubs of Shri Jayachamrajendra zoological gardens respectively were included in each plate. Sensitized RBCs in PBS served as antigen control. The antibody titre was expressed
as the reciprocal of highest dilution giving complete agglutination. Samples showing a titre of 1:32 and above were considered as positive.

The standardization of PHA test was carried out with glutaraldehyde treated sheep erythrocyte using known positive serum, known negative serum and diluted antigen.

3.2 Polymerase chain reaction (PCR)

3.2.1 Extraction of DNA by Frozen blood genomic DNA spin-100 (Catalogue no: RKT 18, 2007-2008, Chromous Bio Tech PVT. LTD, Bangalore)

The Genomic DNA was isolated from the frozen blood sample according to the Frozen Blood genomic DNA spin-100n protocol as described below.

a. Exactly 250 µl of frozen blood was taken into a 2ml vial.

b. To this, 750 µl of 1X frozen lysis buffer was added and vortexed thoroughly for one minute.

c. The mixture was kept at RT for 5 minutes with intermittent vortexing ensuring that at least 70 -80 % of the clot was suspended.

d. The vial was spun at 13000g for 1 minute at RT. The supernatant was discarded till the last drop without disturbing the pellet.

e. 1ml of 1X frozen blood extraction buffer was added to the white pellet. The pellet was broken by vortexing or repeated pipetting and later the vial was placed at 65°C for 15 min.

f. The sample was spun at 13000 g for 2 minutes at RT. The clear supernatant (600 µl each time) was loaded on to the spin column.

g. The spin column was spun at 13,000g for 1 min at RT and the content in the collection tube was discarded. The column was placed back in the same collection tube.

h. 500 µl of 1X wash buffer was added to the column and it was spun at 13,000g for 1 min at RT. The content in the collection tube was discarded and column was placed back in the same collection tube.

i. Step h, was repeated again
j. The empty column was spun at 13,000g for 3 minutes at RT.

k. The spun column was placed in a fresh vial.

l. 35 µl of warm elution buffer was added which was kept at 65°C into the spin column.

m. The vial was kept along with the spin column at 65°C for 1 minute and spun at 13,000 g for 1 minute at RT.

n. Step l and m were repeated, the elute was collected in the same vial and stored at -80°C for further use.

3.2.2 Determination of purity and yield of the DNA samples

The purity and concentration of the DNA extracted from clinical material and biological inoculation were estimated by automated UV spectrophotometry. In brief, 10 µl DNA sample was dissolved in 0.99ml of sterile distilled water. The diluted DNA was transferred into one ml microcuvette and Optical Density (OD) was checked at 260 and 280 nm. Sterile distilled water was used as blank.

3.2.3 DNA confirmation by Agarose gel electrophoresis

The DNA extracted was checked for purity by electrophoresis on 0.8 percent agarose gel.

3.2.3.1 Procedure

a. Required quantity of agarose was weighed and dissolved in proportionate volume of 1X TAE buffer and heated in a microwave oven for one and half minutes.

b. The gel tray was sealed at both ends. The comb was placed into gel tray in proper position, Ethidium bromide 0.4 µg /ml was added to the molten agarose when it cooled to 50°C and poured into the gel tray carefully, avoiding air bubbles. The combs were removed carefully after solidification.

c. The gel tray was kept in the electrophoresis tank and IX TAE buffer was poured to submerge the gel in the tank.

d. The DNA samples were mixed with 1/6th volume of 6X loading dye and carefully loaded into the wells using micropipette.

e. The electrophoresis was carried out at 5 V/cm at RT till the dye had reached the end of the gel and the DNA bands were visualized with a UV transilluminator.
3.2.4 **Detection of *Trypanosoma evansi* infection by molecular methods**

The primers for the present study were procured from Bioserve Biotechnologies (India) Pvt Ltd, Hyderabad. The primer pair (Urakawa *et al*. 2001) was used to amplify at 488bp fragments.

One hundred µl of sterile double distilled water was used to dissolve each lyophilized primer and it was stored at -20°C as stock. The working dilutions were made from the stock solution by mixing 10 µl of stock with 90 µl of sterile DDW.

**Nucleotide sequences.** Forward primer: 5’-GCC,ACC,ACG,GCG,AAA,GAC-3’

Reverse primer: 5’-TAA,TCA,GTG,TGG,TGT,GC-3’

The PCR mix was prepared with Nuclease free water (NFW), Master mix, Template-DNA extracted from blood, 100 bp DNA ladder and Taq Polymerase.

3.2.5 **Amplification of *T.evansi* variable surface glycoprotein gene (VSG) from DNA**

The PCR was carried out in standard 25ml reaction volume for variable surface glycoprotein gene (VSG) target using the published primer pair (Urakawa *et al*. 2001) following standard protocol. A typical reaction containing the following reagents was performed in a 0.2 ml PCR tube.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free water (NFW)</td>
<td>9.0</td>
</tr>
<tr>
<td>Forward primers (20 p moles)</td>
<td>0.5</td>
</tr>
<tr>
<td>Reverse primers (20 p moles)</td>
<td>0.5</td>
</tr>
<tr>
<td>Template DNA</td>
<td>2.5</td>
</tr>
<tr>
<td>Master MIX</td>
<td>12.5</td>
</tr>
<tr>
<td>Total reaction volume</td>
<td>25</td>
</tr>
</tbody>
</table>

The cycling conditions used for amplifying were as follows:

- **Step 1** Denaturation 94°C for 5 min
- **Step 2** Denaturation 94°C for 1min
- **Step 3** Annealing 54°C for 1 min
- **Step 4** Elongation 72°C for 1min

From Step 2 to Step 4 was repeated for 40 cycles

- **Step 5** Final elongation 72°C for 10 min.
3.2.6 Agarose gel electrophoresis of amplicons

Procedure

a. 1.6% molten agarose was prepared in TAE buffer by heating in the microwave oven for one and half minutes.
b. The comb was placed into sealed gel tray in proper positon, Ethidium bromide 0.4 μg /ml was added to the molten agarose when it cooled to 50°C and poured into the gel tray carefully, the combs were removed carefully after the solidification of gel.
c. The gel tray was held in electrophoresis tank filled with IX TAE buffer.
d. The PCR product as mixed with 1/6\textsuperscript{th} volume of 6X loading dye and carefully loaded into the wells using micropipette.
e. The PCR product as loaded in parallel with 100 bp DNA ladder plus molecular weight marker.
f. The electrophoresis was carried out at 5 V/cm at RT till the dye had reached the end of the gel and the bands were visualized with a UV transilluminator.

3.3 Sequencing of Polymerase Chain Reaction products

3.3.1 Polymerase chain reaction

The PCR was carried out for DNA extracted from Lion, Tiger and Leopard isolates as described in section 3.2. The PCR products were stored at -80°C until further use and were sequenced at the Bangalore Genei, Bangalore.

3.3.2 Sequence analysis

The nucleotide sequence data obtained were analysed and edited by BLAST and Clustal W method using Meg Align program of DNA STAR Software.

The \textit{T.evansi} isolates in this study were compared and analysed with sequence data available in Genbank. Additional analysis (Percentage similarity/ difference) in nucleotide sequence and phylogenetic tree was identified with the isolates.