II REVIEW OF LITERATURE

The literature was reviewed on trypanosomosis in wild animals in relation to prevalence, serological and molecular studies and genetic comparison of trypanosome isolates. *Trypanosoma evansi* infection is widely prevalent in the different species of domestic and wild animals.

2.1.0 Prevalence of trypanosomosis in wild animals.

2.1.1 Conventional methods of diagnosis of *T.evansi*.

2.1.1.1 *T.evansi* in wild carnivores

The occurrence of *Trypanosoma evansi* in the form of outbreak was reported by Sinha *et al.* (1971) in tigers, jaguars and leopards, at the Zoological Garden, Calcutta India. Out of the total 7 cases, 2 tigers and 1 jaguar proved to be positive for *T.evansi* infection based on examination of smears from heart blood. Four animals died suddenly one after another without showing any symptoms and gave no clue about the etiology. But, these cases were examined 6-12 hours after death. It was possible that the parasite in the blood disintegrated by the time postmortem was done.

Sen Gupta (1974) reported *Trypanosoma evansi* infection at the Zoological Garden, Alipore, Calcutta, in 2 White tigers, 2 Royal Bengal, 2 wolves, and 1 clouded leopard. Out of which 1 wolf, 1 Royal tiger and a clouded leopard died and blood examination revealed trypanosomes.
An outbreak of Surra in tigers of the New Grand Circus stationed at Vijayawada was reported by Govinda Reddy et al. (1975) with the history of high temperature (106°F to 107°F), anorexia and not responding to instructions and the blood smear revealed trypanosomes.

Dasgupta et al. (1979) reported an outbreak of *T.evansi* in tigers (*Panthera felis altaica*) and leopards (*P.pardus*) in the Himalayan Zoological Park in Darjeeling, India. The tigers and the leopard recovered after treatment with quinapyramine.

Choudary et al. (1986) reported a case of sudden death in a one and half years old male tiger cub (*Panthera tigris*), belonging to Nehru Zoo Park, Hyderabad which had high temperature (104°C) and was unable to come to its enclosure. On post mortem examination the heart muscle impression smear revealed *T. evansi* organisms.

Renuka Prasad et al. (1991) demonstrated *T.evansi* in five captive tigers of the Bannerghatta National Park, Bangalore based on blood smear examination.

Ramachandraiah et al. (1994) reported a case of trypanosomosis in a male tiger from a circus in Andhra Pradesh which had suddenly fallen ill with dullness, dyspepsia, pyrexia, rapid pulse and respiration, diarrhea and swelling of the hind limbs. The blood smears revealed the presence of trypanosomes.

A case was reported by Ramachandraiah et al. (1995), of a male tiger belonging to the New grand circus at Chittoor that had suddenly fallen sick. The clinical examination revealed dullness, dyspepsia, pyrexia 104.6°F, rapid pulse, rapid respirations, diarrhea and swelling of hind limbs. The blood smear examination showed *Trypanosoma evansi* organisms. The animal recovered successfully when treated with 2.5 g of Berenil along with supportive therapy.

An outbreak of Surra in a circus tiger in five adults and four cubs was reported by Bhaskara Rao et al. (1995) that belonged to the National circus organized at Kakinada (AP), India. The microscopic examination of blood smears revealed *T.evansi* infection. The animals showed symptoms of rapid pulse, rapid respiration and temperature ranging from 104°F-106°F.

Arias et al. (1997) examined 559 Capybaras (*Hydrochoerus hydrochaeris*) during the slaughtering season of February and March 1991 in two areas in Venezuela. *T.evansi* antibodies were detected in 48
animals by the microcentrifugation technique and 279 of the 559 Capybaras revealed antibodies in the immunofluorescence test. It was concluded that Capybaras could be important in the epizootiology of *T.evansi* in enzootic areas where they coexist with domestic horses, cattle and other wild animals.

Upadhye and Dhoot (2000) reported trypanosomosis in 9-year old male tiger maintained in a zoo in Maharashtra State. The tiger showed symptoms of anorexia, constipation, lethargy and convulsions, which was confirmed by blood smear examination.

Dakshinkar *et al.* (2002) reported trypanosomosis in a jungle cat (*Felis chaus*) aged one year maintained in captivity at Maharajbhag Zoo, Nagpur. Clinical examination revealed bilateral corneal opacity with ocular discharge and body temperature of 101°F. Peripheral blood smear examined revealed *T.evansi* (10-12 per field).

Utkarsh-Shukla (2002) reported trypanosomosis in a wolf (*Canis lupus*) belonging to Lucknow zoo.

Murali Manohar *et al.* (2003) reported *T.evansi* infection in an apparently healthy male Royal Bengal tiger; aged about 12 years which belonged to Arignar Anna Zoological Park, Chennai that had died suddenly. Blood smears collected immediately after death of the animal revealed *T.evansi*. But, the smears collected after 6 hours did not reveal organisms. Enlargement of spleen and lymph nodes, an increase of pericardial fluid, congested liver, spleen, kidney, lung and brain were noted. They observed, diffuse infiltration of lymphocytes, macrophages and plasma cells in liver, spleen, kidneys, lungs, stomach, intestine and testes.

Samantray *et al.* (2003) reported the death of 12 tigers in Nandankanan Zoo in Orissa, which died due to trypanosomosis in June-July 2000 despite efforts to save them.

Trypanosomosis in an adult circus female tiger was reported by Devasena and Shobhamani (2006) which had clinical signs of pyrexia (104°F), depression, weakness, lacrimation, watery nasal discharge, rapid respiration and edema of the abdominal region which was presented at the Veterinary Polyclinic, Chitoor, Andhra Pradesh. The blood smear revealed trypanosomes and microcytic normochromic anaemia.

**2.1.1.2 *T.evansi* in wild herbivorous animals.**
Clausen (1981) examined the blood of black Rhinoceros (*Diceros bicornis*) which were captured. Blood samples were collected from 39 rhinoceros from Tanzania, Kenya and Nairobi during the period 1968 to 1970 and trypanosomes were found in blood smears from 7 of 39 (18%) animals which were identified as *T. brucei*. No correlation between biological and serological tests was found.

The host-parasite relationship of various strains of *T. evansi* in India was studied. Raisinghani *et al.* (1981) reported on the behavior of the camel strain of *T. evansi* in experimentally infected Jersey x Rathi cross-bred calves and found that cross-bred calves were refractory to infection.

*Trypanosoma evansi* infection in red deer was reported from China by Gu *et al.* (1982). In the winter of 1979, 124 deaths occurred at a deer farm in Tengchong Country. Blood smears from the dead and affected animals revealed trypanosomes which were identified as *T. evansi*. Animal inoculation showed that the parasite was pathogenic to horse, cattle, sheep, mice and guinea pigs.

Chen *et al.* (1983) reported deaths at a deer farm in South China in the winter of 1978 in which 70 of the 360 deer died after showing symptoms of progressive emaciation, anaemia, oedema and nervous signs. Inoculation of mice revealed the presence of trypanosomes 6-15 days after infection.

Pathak *et al.* (1988) reported the occurrence of trypanosomosis in two male adult sambars of two local deer parks, of the Maharashtra state forest department which were presented for necropsy.

Lefebvre *et al.* (1997) reported trypanosomosis in woodland Caribou (*Rangifer tarandus caribou*) from three areas of northern Alberta, Canada. Trypanosome (*Megatrypanum*) sp were present in 41 of 49 cultures, but none was detected in fresh blood.

Singh (1998) carried out a 6-year epidemiological survey on *T. evansi* infection among free living wild and domestic animals in a nature reserve of Rajasthan State. Outbreaks of trypanosomosis were reported in May 1993, August 1994 and June 1997 among spotted deer (*Cervus axis*), sambar (*C. unicolor*), feral cattle and domestic animals. *T. evansi* was present in blood and lymph smears from 23 of 77 spotted deer, 37 of 83 sambar, and 98 of 117 feral cattle, 87 of 155 buffaloes, 100 of 119 sheep and goats, and 23 of 53 camels. The parasites were not found in any of the 8 tigers (*Panthera tigris*), 15 leopards (*P. pardus*).
hyaenas (*Hyaena hyaena*), 17 nilgai (*Boselaphus tragocamelus*), 32 wild boars, 12 jackals (*Canis aureus*), 17 gazelles (*Gazella sp.*) and 13 black bucks (*Antelope cervicapra*) which were examined.

Tuntasuvan *et al.* (2000) detected *T.evansi* in the brain of naturally infected hog deer that ranged free on a farm in Samut Prakarn province, Thailand, which died after showing nervous signs between September 1997 and February 1998. They demonstrated organisms by streptavidine-biotin immunohistochemistry.

Sinha *et al.* (2002) reported Surra in a female elephant of Sanjay Gandhi Zoological Park, Patna which had left lateral recumbency, no movement of trunk and tail, gripping pain, tears in eyes, presence of many ecchymotic foci on conjunctivae with recurrent fever (99.6-100.8°F) followed by mild subnormal body temperature (96.6°F). The animal was suffering from loss of appetite and anaemia, Wet blood preparations were examined for live organisms and were declared positive for *T.evansi* infection.

Harish *et al.* (2004) reported the incidence of acute to sub-acute trypanosomosis in horses from Kampli village, Bellary district of Karnataka state. The herd comprised of 60 horses of which 52 animals exhibited acute form. The severity of the disease resulted in death of 22 animals. Blood smear examination showed large number of organisms and high population of blood sucking tabanid flies were observed in the area.

Abdel-Rady (2008) conducted epidemiological studies in 193 camels (*Camelus dromedaries*) in Egypt for *Trypanosoma evansi* infection. The Giemsa stained blood smear revealed eight camels to be positive and haematocrit centrifugation technique detected 12 to be positive, card agglutination test (CATT) indicated 84 to be positive and DNA amplification by polymerase chain reaction (PCR) could identify 110 as positive. The primers yielded a 177 bp PCR product as specific for the parasites. It was concluded that PCR technique was accurate, more sensitive and specific for the diagnosis of trypanosome-infected camels compared to other parasitological techniques.

### 2.1.2. Serological diagnosis of *T.evansi*

#### 2.1.2.1 Serological tests for *T.evansi*
The serological tests are reliable for diagnosis of trypanosomosis in animals, as they react specifically to *T.evansi* antigen. The literature on various serological tests adopted for diagnosis of *T.evansi* infection has been reviewed.

Diagnosis of Surra in camels by the passive haemagglutination test was adopted by Jatkar and Singh (1971). This test was used to study antibody titers from *T.evansi* infected and non infected camels from Bikaner (Rajasthan). The positive cases of *T.evansi* infected animals revealed titres of 1: 40 and above. The titres of 1:20 was considered doubtful.

The passive haemagglutination test was conducted by Shen (1974) which was found to be highly specific and sensitive for detection of antibodies in goats experimentally infected with *T.evansi*. The titres of 1:20 and above were positive, 1:10 was regarded as doubtful, while lower titers were negative. This test was recommended for screening in the surra eradication programme.

Verma and Gautam (1977) evaluated three serological tests viz., passive haemagglutination test, gel diffusion test and Indirect fluorescent antibody test for detection of experimental *T.evansi* infection in 10 buffalo and 5 cow calves. The PHA test was reliable and sensitive. The test was found positive on the 4th day post infection and maximum positive reactions were found between 6th and eight days post infection. Precipitating antibodies were detected on 8th day post infection in 2 infected buffalo calves and in the remaining 13 animals between 12th and 20th day. The Indirect fluorescent antibody test detected antibodies in one buffalo calf on 8th day post infection and in other 14 animals between 12th and 16th day. The intensity of fluorescence increased in later stage of the disease.

Luckins (1977) reported that the micro ELISA detected trypanosome specific antibodies in cattle infected with *T.vivax, T.congolense* or *T.bruceti*. There were significant differences in microELISA results obtained from samples of infected and non infected cattle. During the course of infection micro ELISA values were found to fluctuate and antibody titre value varied in individual animals. The test did not distinguish infection between *T.vivax, T.bruceti* and *T.congolense*. There was no cross reaction between trypanosome antigens and serum samples from cattle infected with *Trypanasoma theileri, Theileria parva, Theileria mutans, Theileria annulata, Babesia divergens* and *Anaplasma marginale* organisms.
Luckins et al. (1979) adopted indirect immuno florescence antibody test, ELISA and IgM assay which gave positive results in 29 (96.7%), 26(86.7%) and 17 (56.7%) of the animals respectively.

Raina et al. (1985) screened sera collected from six buffalo calves before and upto 70 days after infection with *T.evansi* by indirect haemagglutination test and capillary tube agglutination test. All pre infective sera were negative but from the seventh day onwards all sera were positive by indirect haemagglutination test. All sera were positive by capillary tube agglutination test from day 21 to day 56 and by the 70th day only 50% were positive. Both tests were specific and no cross reactions were detected with other protozoan diseases.

Hu et al. (1985) conducted the indirect haemagglutination test to study *T.evansi* infection in experimentally infected buffaloes. One of three experimentally infected buffaloes was positive by IHA on the 7th day and the other 2 were positive on the 21st day. All of them remained positive for the whole course of infection.

Shen et al. (1985) carried out Enzyme linked immunosorbent assay for diagnosis of *T.evansi* infection in buffaloes at Shanghai (China). ELISA proved to be the satisfactory method in the diagnosis of trypanosomosis in buffaloes caused by *T.evansi*. In a comparative study involving ELISA, IHA and CF tests, the rates of detection were 96.2%, 78%, and 82.3% respectively.

Discontinuous counter-immunoelectrophoresis was used to detect *T.evansi* antibodies in experimentally-infected buffalo calves. Raina et al. (1986) found that the method was simple, reproducible, rapid and sensitive. The results could be read within 15 minutes and compared favourably with results obtained with the agar gel precipitation test.

Bajyana Songa and Hamers (1988) assayed a variable antigen type (VAT) of *T.evansi* by card agglutination test and compared it with a commercially available test trypanasoma kit in infected serum from pigs, cattle, buffaloes, camels and *T.gambiense* infected human sera. The results were found more sensitive.
Novikov (1988) adopted IHA for serological diagnosis of trypanosomosis in 46 camels in Turkmenia with freeze dried erythrocytic antigen and 14 samples out of 46 gave titre of 1:16, and 5 serum samples gave a titre of 1:256. The test was more reliable than formaldehyde gel test.

Diall et al. (1992) evaluated mono and poly clonal antibody based antigen detection immunoassays for diagnosis of *T.evansi* infection in the dromedary camel in Kenya and Mali. The results indicated that these immunoassays were six times more sensitive than the haematocrit centrifugation technique.

Blood samples from 240 camels were examined for trypanosome infection by Pathak et al. (1993). Of these 18 were found to be infected by the wet blood Giemsa stain technique, 76 camels were found to be positive for *T.evansi* antigen using double antibody sandwich enzyme-linked immunosorbent assay (ELISA), which was found to be more useful method for the detection of current infection.

Saseendranath et al. (1995) adopted passive haemagglutination test for diagnosis of *T.evansi* infection in sheep. The PHA could detect *T.evansi* specific antibodies at 21 days post infection. The antibody titre tended to increase from 3rd week of infection in subacute and chronic cases, and declined by the 13th week post infection.

Bengaly et al. (1995) reported that ELISA for antigen detection showed 65.8% sensivity and 98% specificity in the naturally occurring *T.evansi* infections. They suggested the combination of Ag ELISA and buffy-coat method for the diagnosis of trypanosomosis particularly in epidemiological study in endemic area.

Olaho-Mukhani et al. (1996) carried out latex agglutination test for diagnosis of *T.evansi* in camels from rift valley province of Kenya. The latex agglutination test detected *T.evansi* infection in 46.3% of animals. They concluded that latex agglutination test could be of help in rapid field diagnosis of *T.evansi* infection in camels.

Pathak et al. (1997) found a good correlation between the positive results obtained by wet blood film, CAT and Ag-ELISA. They inferred that CAT could be used to study the seroprevalence of *T.evansi* with great ease; however, trypanosome antigen detection gave a more accurate idea of the prevalence of *T.evansi* in an endemic area.
Ghorui and Samanta (1998) in their experimental study in rabbits indicated that counter immuno-electrophoresis test could detect *T.evansi* antibodies as early as seven days post infection.

Seroprevalence study of *T.evansi* infection in bovines was studied by Krishnappa *et al.* (2002) with passive haemagglutination test. Out of 608 sera samples screened, 170 (27.98%) were positive for PHA test with antibody titres ranging from 32 to 2048. 1:32 was taken as cut off value to declare PHA as positive.

Oyieke (2003) reported the occurrence of dromaderine trypanosomosis in selected northern Kenya herds by using the enzyme linked immunosorbent assay (ELISA), mouse inoculation (MI) and blood smear techniques (BS). The ELISA results indicated current or past trypanosomal infection with prevalence rate of 72-95%. MI and BS techniques revealed the current infection rates up to 19.2 and 11.5%, respectively. The infection rates were significantly elevated during the wet season which was observed based on the MI diagnostic technique.

A cross-sectional study was conducted by Delafosse and Doutoum (2004), to estimate the prevalence of *T.evansi* infection in herds of camels from the eastern area of Chad. Blood samples were collected and examined for the presence of *T.evansi* using an antibody (CATT/*T.evansi*) and a parasite detection test (BCT). The apparent prevalence was 5.3% using BCT and 30.5% with CATT.

Serum samples of apparently healthy dromedary camels from Surra endemic areas of Rajasthan in and around Bikaner were screened by Shahardar *et al.* 2004 by Ouchterlony’s double immunodiffusion (DID) and counter immuno-electrophoresis (CIEP) tests for presence of anti-bodies with *T.evansi* detergent solubilized antigen. Out of 50 and 107 serum samples examined by DID and CIEP, antibodies to *T.evansi* were detected in 2% and 19.62% camels respectively.

### 2.1.2.2 Mouse inoculation and maintenance of *T.evansi* isolates.
Trypanosomes are maintained through serial in vivo passage in a wide range of laboratory animal hosts and also by cryopreservation (Lumsden et al., 1973). Luckins et al. (1978) established that the prepatent period in mice was dose-dependent and directly related to host resistance.

The effect of environmental temperature on T.evansi infection in mice was studied by Mathur et al. (1976). The infectivity and virulence was markedly reduced in mice maintained at an ambient temperature of 35°C when compared with that at room temperature (22-26°C), based on 3 different strains of T.evansi.

In three different places viz, Tanzania, Kenya and Nairobi, Clausen (1981) screened 39 black rhinoceros for trypanosomes. Five of 26 blood samples of rhinoes, injected into mice and rats yielded isolates.

In comparative studies on diagnosis of equine trypanosomosis by six parasitological methods, Monzon et al. (1990) in the subtropical area of Argentina, demonstrated T evansi in 52 samples. Mouse inoculation gave a sensitivity of 88.2%, Giemsa-stained smears (45.6%), wet blood films (53.8%), Strout’s concentration method (46.1%), haematocrit centrifuge technique (71.1%) and buffy coat method (63.4%). They concluded no single method alone was totally effective. The haematocrit centrifuge technique, mouse inoculation and giemsa-stained smears were proposed as the most effective diagnostic combination.

Sarmah (1998) observed the survivability of T.evansi organisms and found them alive in the heart blood for at least 10 hr after death of the experimentally infected rats which was infective to mice on subsequent inoculation. Examination of Giemsa’s stained blood smear prepared 12 hr after death of rats revealed total degeneration of parasites. Liver and spleen impression smears showed no trace of the parasite.

A study was conducted by Singh et al. (2003) in the villages of the Bikaner district, in Rajasthan, where Surra in camels is endemic. Camels from the Amarpura and Nada villages were examined between July 2002 and May 2003. A volume of 1.0 ml of blood was inoculated intraperitonelly into healthy albino mice and monitored up to 60 d post inoculation. The study revalidated the value of mice inoculation as a diagnostic technique, as it resulted in a 50% increase in animals found to be positive.

Silva et al. (2007) experimentally infected Trypanosoma evansi orally to rats and mice. They were fed 0.2 ml of contaminated blood with 107 trypanosomes ml⁻¹ by using probe. It was observed in rats that the
prepacy period varied from 19 to 25 days and the period of parasite detection and animal’s death was an average of 12.7 days. The mice were evaluated for 60 days but did not show infection. It was concluded that the rats could be orally infected with *T.evansi* but not mice.

Lisboa et al. (2007) studied biological heterogeneity in the isolates derived from wild hosts. The course of experimental infection of Swiss mice with 95 sylvatic *Trypanosoma cruzi* isolates included in TCI or TCII genotype was characterized. A significant biological heterogeneity was observed in both isolates. Higher biological heterogeneity was observed in *T.cruzi* II isolates derived from *L.rosalia* from Atlantic coastal rain forest. TCII isolates derived from marsupials resulted in very similar infection profile in Swiss mice.

**2.1.2.3. Isolation and purification of Trypanosomes**

Soltys et al. (1969) reported slow and high speed centrifugation of blood infected with *Trypanosoma brucei* for the concentration of trypanosomes.

Lanham and Godfrey (1970) employed Diethyleaminoethyle (DEAE) Cellulose method for eluting trypanosome parasites from infected blood. Experimental animals such as rats, rabbits, mice, guinea pigs, monkeys and other mammalian species were utilized for sub inoculation. Large quantity of pure trypanosomes could be produced, for pure antigen preparation for immunodiagnostic techniques.

Gill (1970) compared various methods for recovering trypanosomes from blood of rats. The recovery of 93.2, 86, 82 and 71% trypanosomes by agglutinating host’s red blood cells with phytohaemagglutinin, anti-rat red blood cell serum produced in rabbits, differential centrifugation and lysis of the red cells with water respectively was found. Comparison of various methods of preparing the antigen revealed that the clarified homogenate prepared by disrupting the trypanosomes with ultrasonic vibrations proved to be the best antigen, followed by acetone-dried organisms, lyophilized whole organisms and glycerinated suspension of the trypanosomes.

Jatkar and Singh (1971) adopted a crude method of purification and isolation of trypanosoma antigen by collecting infected camel blood with a high parasitaemia. The parasites were separated and
suspended in glucose and left at room temperature for 24 hours, later it was preserved at 4°C and used as parasite antigen in passive haemagglutination test.

Taylor *et al.* (1974) modified the solution of Lanham buffer for *T. congolense* isolation on Diethyl-52 columns which prevented impairment of activity and infectivity of *T. brucei*.

Luckins (1977) used various strains of trypanosomes for the preparation of antigens for the ELISA test. Infected blood was collected from mice with fulminating parasitaemias and parasites were separated on a column of DEAE-Cellulose by adopting the method of Lanham and Godfrey (1970). The separated trypanosomes were washed three times with PBS (pH 7.4) and stored at -79°C until required. Several batches of this material were pooled for subsequent processing. Pooled antigens were diluted ¼ with ice cold PBS and then subjected to 20s ultrasonification at maximum amplitude on an MSE 100 w ultrasonic disintegrator. The resulting suspension was centrifuged and the supernatant frozen at -79°C as stock antigen.

Jatkar and Purohit (1977a) used dog serum which completely agglutinates red blood cells of rats and mice used for separation of *T. evansi* from the blood of infected rats and mice for preparing antigen.

Red blood cell stroma antigen was prepared by Jatkar and Purohit (1977 b) from blood collected with EDTA from a horse infected with *T. evansi*. Few drops of acetic acid was added and washed with distilled water and normal saline by centrifugation till no haemoglobin was seen. The stroma prepared was diluted to 1% solution in normal saline and sonicated, the supernatant was collected and concentrated in a freeze dryer to half of its volume.

Purohit and Jatkar (1979) collected blood from horse which was experimentally infected with *Trypanosoma evansi*. The blood was collected in 2% EDTA phosphate buffered saline on 11th and 24th days post infection, where peak parasitaemia was observed. The parasites were separated by differential centrifugation and antigen was prepared.

Rosen *et al.* (1979) isolated and purified *Trypanosoma congolense* which were grown in rats. An increase in the number of parasites was achieved by replacement of blood by lactated ringer’s solution with 5% glucose as the rats were bled from the abdominal aorta. The method of DEAE Cellulose could separate
trypanosomes from 95% of erythrocytes by differential centrifugation. The purification was effective by removing contamination as they adopted DEAE cellulose column method.

Ahuja et al. (1985) adopted phytohaemagglutinin for purification of *T.evansi* from the blood of experimentally infected rats. Exposure of *T.evansi* to prolonged and/or high concentrations (4-14 mg/ml blood) of phytohaemagglutinin killed and degenerated the trypanosomes, quick removal of the trypanosomes from phytohaemagglutinin medium and suspension in phosphate saline glucose (pH 8.0) medium prevented killing and degeneration of trypanosomes.

Lohr et al. (1986) propagated *T.evansi* in rats and isolated by using DEAE cellulose method of Lanham and Godfrey (1970). After ultrasonic treatment of eluted parasites, the supernatant was used as antigen in complement fixation test (CFT).

Srivastava et al. (1988) used a column of 5 cm height and 2 cm diameter in DEAE cellulose column chromatography for isolation and purification of trypanosomes from infected rat blood, using heparin as anticoagulant and obtained 80-90% trypanosomes.

Monzon (1993) separated trypanosomes from blood cells by centrifugation for 7 min at 754g. After washing three times in PBSG (phosphate-buffered solution pH 7.3 containing 1% glucose), they were weighed and suspended in a 0.2% trypsin solution and stabilized with formalin for detection of *T.evansi* infection in horses with direct agglutination test.

Veer Singh et al. (1995) prepared whole cell lysate antigen from trypanosomes collected from experimentally infected albino rats by separation of DEAE cellulose. The parasites were sonicated at 150 W for 3mins, with three to four cycles using an ultrasonic disintegrator. The supernatant fluid was collected and the protein content adjusted to 1.0 mg ml\(^{-1}\) with PBS pH 8.0 determined by Lowry’s method. The antigen of different stock was stored at -20 °C.

Amato Neto et al. (1996) applied the use of quantitative buffy coat (QBC) method which is used as malaria diagnostic technique, for the laboratory diagnosis of *Trypanosoma cruzi* infection used in an experimental mouse model. The sensitivity of the method was assessed with 1/20 dilution and 200 parasites
were detected per capillary tube. The dilutions of 1/10, 240 resulted in detection of a single parasite. The QBC method was found to be effective for the diagnosis of *T.cruzi* infection in mice.

Jithendran and Rao (1999), separated trypanosomes from a highly parasitaemic rat blood by DEAE-cellulose chromatography. Whole cell lysate antigens of *T. evansi* were prepared under different solubilising conditions. Buffers used in lysis procedures contained PMSF (100 mM) TLCK (10mM) to minimize proteolysis of VSGs. Sonication of whole cell preparation of *T.evansi* at cell concentration of 3 to 5x10^8 ml^-1 gave good harvests of soluble antigen at a protein concentration of 23.7 mg ml^-1.

*Trypanosoma equiperdum* and *T. evansi* were purified by three or four cycles of low-speed centrifugation and final filtration was done through DEAE cellulose. The purified trypanosomes were used for comparative biochemical and immunological studies by Giardina et al. (2003).

Gu-ZeMao et al. (2006) isolated *Trypanosoma monopteri* from blood of naturally infected fish (*Monopterus albus*). Whole blood was obtained from the fish caudal vein using a 2ml heparinized syringe. Most of the erythrocytes were removed by centrifugation at 2.9 x 103 r/min and 15 min with 51% percoll reagent containing PGSS. The collected suspension contained trypomastigotes, white cells, platelets and few red blood cells. To remove remaining blood components, DEAE-52 cellulose column equilibrated with PSG was employed. The recovery of blood trypanosomes was 88.98 and 83.80% respectively and was morphologically normal and actively motile.

### 2.2. Molecular diagnosis of *T.evansi*.

Comparative studies on the sensitivity of PCR and microscopic examination for the detection of *T.evansi* in experimentally infected mice was studied by Ijaz et al. (1998). During acute phase of infection, parasites were detected by PCR 3 days earlier than microscopy. The infected mice were found consistently positive by PCR during the chronic phase when parasites could not be demonstrated using microscopy. They suggested PCR could be used for the diagnosis of camel trypanosomosis during both acute and chronic phases of infection and for use in the evaluation of treatment.

Omanwar et al. (1999) adopted polymerase chain reaction (PCR) for detection of *T.evansi* by DNA amplification from crude blood sample collected and blotted on to a previously autoclaved filter paper. The
PCR test was capable of detecting *T.evansi* parasites at low parasitaemia and from samples of carrier animals. They concluded that PCR amplification of DNA using parasite-specific primers represented a potentially powerful tool for epidemiological studies in animal trypanosomosis.

Polymerase chain reaction was applied as a diagnostic tool for detecting trypanosomes in naturally infected cattle in Burkina Faso by Solano *et al.* (1999), and found it to be an efficient tool to estimate the prevalence in affected area.

Detection of *T.evansi* in dromedary camels by polymerase chain reaction to rDNA target was standardized by Ahmad (2000). The analysis of the PCR product on 1.4% ethidium bromide stained agarose gel revealed specific amplification product of 518 bp DNA fragment from the genome of *T.evansi*.

Mugittu *et al.* (2001) used the polymerase chain reaction (PCR) and deoxyribonucleic acid (DNA) probes to characterize trypanosomes from cattle in Morogoro region of Tanzania. Blood samples collected from 390 beef and dairy cattle in selected farms in Morogoro region were examined for presence of trypanosomes using the buffy coat technique and blood smear. Fifty two animals were found infected by PCR and trypanosome DNA could be detected in 27 (43%) out of 62 samples that were parasitologically negative.

Masiga and Nyang’AO (2001), characterized trypanosomes from field infections of camels using PCR and the procyclic transformation test (PTT). The *invitro* transformation was used to distinguish between *T.brucet* and *T.evansi*. Parasites were passaged in mice and DNA extracted from trypanosomes isolated from mouse blood. DNA primers specific to *T.evansi* type A, *T.congolense*, *T.vivax* and a satellite DNA sequence specific for the subgenus Trypanozoon were used to screen a total of 80 samples. *Trypanosoma evansi* was detected in 76% of the isolates, confirming it to be the most important species causing trypanosomosis in camels in Kenya.

Dethie Faye *et al.* (2001) examined blood of equines in Gambia for trypanosomes by the buffy-coat (BC) method and polymerase chain reaction (PCR). PCR detected seven times higher number of positive cases compared to the BC method.
Desquesnes and Davila (2002) achieved the several levels of specificity from subgenus to species, subspecies and even types. Random priming of trypanosome DNA which allowed “isolate specific” identification suggested for regular diagnosis of animal trypanosomosis, the sensitivity of PCR increased with the advancement of technologies for sample preparation, to reach a level of 1 trypanosome/ml of blood, of field samples. The sensitivity was two to three times higher than microscopic observation of the buffy coat.

Njiru et al. (2004) detected *T. evansi* in camels using polymerase chain reaction (PCR) and card agglutination test (CATT/*T. evansi*) tests in Kenya. A total of 549 camels were screened for infection using microhaematocrit centrifugation technique (MHCT), PCR and the CAT/*T. evansi*. There was a significant difference in the prevalence wherein MHCT was significantly lower than those of PCR and CATT/*T. evansi*. They also observed poor association between the published clinical signs, seropositivity, PCR and MHCT and found a higher agreement between farmers classification of disease with the PCR test.

Singh et al. (2004) evaluated parasitological, serological, and DNA amplification methods for diagnosis of natural *T. evansi* infection in 217 camels from different areas of western Rajasthan State, India, examined from July 2002 to May 2003. PCR revealed a specific 227 bp band in positive samples. The intensity of PCR bands was variable in different test samples depending upon the level of infection in the test samples. The history of clinical signs correlated with positive serological status in ELISA as well as trypanosome DNA detection by PCR.

Standardization of polymerase chain reaction for diagnosis of trypanosomosis in captive wild animals was carried out by Shailaja et al. (2005). Giemsa stained blood smear was scraped and used for this technique, genomic pair used to amplify 488 bp fragments was used. Out of 44 samples 11 were found to be positive by microscopic examination and 14 to be positive by PCR method which was concluded to be a sensitive method for diagnosis.

Ravindran et al. (2008) compared sensitivity and specificity of PCR and blood smear for the detection of *T. evansi* in camels (n=61), donkeys (n=44) and dogs (n=26). Out of 131 blood samples tested, 26 samples (21 camels, 3 donkeys and 2 dogs) were detected positive by PCR. Blood smear examination revealed *T. evansi* only in two camels.
Ghorui et al. (2009) reported the use of PCR for the detection of *T.evansi* in camels and equines. PCR detected infections in 14 out of 153 camels and 4 out of 11 equines. PCR using the VSG gene primer pair revealed the same infection.

Shahardar et al. (2009) used polymerase chain reaction for detection of *T.evansi* in Indian dromedary camels using ribosomal DNA amplimers based on structural 18S and 5.8S ribosomal DNA sequences specific for kinetoplastida taxon. Out of 10 blood samples tested six of them were found positive reaffirmed the suitability of PCR as sensitive diagnostic tool. The blood sample assessed showed a distinct PCR product of expected size of 518 bp, but some non-specific amplimers in the molecular size of 200-400 bp were seen in some samples, Due to highly conserved nature of these DNA sequences in ribosomal DNA in various species. This needs further studies on the DNA fidelity of the segments co-amplified by hybridization technique using species-specific DNA probes.

2.3. Comparision of isolates of *T.evansi*.

Intra-species differentiation of *T.evansi* by DNA fingerprinting with arbitrary primered polymerase chain reaction was developed by Wantanapokasin et al. (1998), in Thailand. Only one out of 10 randomly designed 12-mer primers generated DNA fingerprint profiles that revealed intra-species differences in *T.evansi*. The AP-PCR method was found to be simple, fast and sensitive to diagnose and characterize the parasites since it did not require prior DNA sequence information.

Biometrical observations on buffalo, bovine and canine strains of *T.evansi* isolated from species in Madras, were carried out by John et al. (1992). The strains varied significantly in total length and width. The buffalo strain had a greater free flagellum, total length and width compared with the canine strain.

Biometrical alteration in a horse isolate of *Trypanosoma evansi* was observed when passaged in laboratory rodents by Davila et al. (1998). They observed major parasite transformation based on increase in the total length as a consequence of an increase in some parasite measurements attributed it to a host change from horse to rodents.

Pareek et al. (1999) studied the polypeptide profile of *T.evansi* isolates of camel and cattle origin. The cattle isolates revealed 11 protein bands with molecular weights ranging from 195 to 26 k Da, while
camel isolates revealed 20 proteins ranging from 180 and 24 K Da. Eight polypeptides were found to be identical in both isolates.

Biological and biochemical characterization of isolates derived from coati (*Nasua nasua*, carnivore, Procyonidae), horses and dogs, of *T.evansi* from Pantanal of Matogrosso- Brazil was studied by Queiroz *et al.* (2000). They observed that biological heterogeneity did not correspond with the biochemical homogeneity observed in *T.evansi* isolates. The variability in virulence was very significant, but not correlated with the host from which it was derived. The data suggested that *T.evansi*, is transmitted among both domestic and sylvatic animals in one single transmission cycle.

Comparative pathogenicity of three genetically distinct types of *T. congolense* in cattle was clinically observed by Bengaly *et al.* (2002). The result indicated clear difference in pathogenicity between the three types of *T.congolense*; the savannah-type infection was virulent while forest-type was of low pathogenicity and the kilifi-type was non-pathogenic.

Tewari (2004) studied genetic polymorphism by RAPD-PCR technique between the isolates of *T.evansi* from dog and camel. 12 potentially informative random primers were used for identification. A similarity co-efficient of 40 to 100 percent was observed between the isolates depending on the primers used.

Amplification of RoTat 1.2 VSG gene by PCR in *T.evansi* isolates in Kenya was done by Ngaira *et al.* (2004). They used purified DNA samples using an established RoTat 1.2 based polymerase chain reaction that would yield a 488 bp product for the specific detection of *T.evansi*. The RoTat 1.2 VSG gene was found absent in some *T.evansi* strains infecting camels in Kenya. The strains that lacked the gene was the *T.evansi* reference isolate KETRI 2479 known to belong to a minor isoenzyme group corresponding to the types Bk DNA minicircle of *T.evansi*.

Genetic heterogeneity in 18 *Trypanosoma cruzi* strains isolated from naturally infected triatomine vectors in northeastern Brazil was studied by Pacheco *et al.* (2005). Multilocus enzyme electrophoresis (MLEE) and randomly amplified polymorphic DNA (RAPD) analyses were used to investigate the genotypic diversity and spread of the *T.cruzi* genotypes in different environments. MLEE clearly distinguished two distinct isoenzyme profiles and RAPD analysis revealed 10 different genotypes to be
circulating in rural areas. The strains were typed as isoenzyme varients 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 plastiware

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. Plastiware 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