V DISCUSSION

The present research was undertaken to know the prevalence of *Trypanosoma evansi* infection in captive wild animals from different regions of Karnataka, based on blood smear examination, serodiagnosis by PHA and parasite DNA amplification by PCR. Effort was also made to get different trypanosome isolates from both domestic and captive wild animals from different parts of the state, for phylogenetic analysis.

The diagnosis of trypanosomosis is usually based on clinical signs and demonstration of the parasites in the blood and in some instances by serological tests. The clinical manifestations are indicative but not specific enough to confirm the disease without the laboratory diagnostic methods. In the presence of high parasitaemia, the examination of wet blood film, stained blood smears and lymphs node aspirates would reveal the trypanosomes. It is recommended in chronic cases such as carrier status, to examine thick blood smears as well as methods of parasite concentration and the inoculation of laboratory animals (OIE, 2000).

5.1.0 Prevalence of trypanosomosis in wild animals.

Despite the fact that trypanosomosis is known to be one of the most important haemoprotozoan disease of wild animals in India, comprehensive work on the epidemiological status and diagnostic methods was lacking. Most of the reports are based on the parasitological diagnostic methods like blood smear staining and response to therapy and in the face of outbreaks or clinical disease.

5.1.1 Conventional methods of diagnosis of *T.evansi*

The first report of *Trypanosoma evansi* outbreak in tigers, jaguars and leopards, at the zoological Garden, Calcutta India was reported by Sinha *et al.* (1971). These animals were proved positive by blood smear examination. In the same *Zoo Sen Gupta* (1974) also detected the organisms on blood examination in 2 wolves, tiger and a leopard. In the present study, blood smear examination of all 113 samples screened did not reveal trypanosomes inspite of getting trypanosome isolates from the leopards and lions from the Tiger-lion safari at Thyavarekoppa, the smears of these animals did not reveal organisms in the blood smear. The animals were active and apparently healthy. There are many case reports on trypanosomosis both in captive wild animals which includes tigers, jaguars,leopard wolves, jungle cat, sambars, spotted deer, hog deer and
elephants which were diagnosed based on blood smear examination and clinical symptoms (Govinda Reddy et al., 1975; Dasgupta et al., 1979; Choudary et al., 1986; Pathak et al., 1988; Renuka Prasad et al. 1991; Ramachandraiah et al., 1994 & 1995; Baskara Rao, et al. 1995; Upadhya and Dhoot, 2000; Dakshinkar et al., 2002; Utkarsh-Shukla, 2002 and Samantray et al. 2003).

The present study is in accordance with the report of Singh (1998) who carried out a six-year epidemiological survey on *T.evansi* infection among free living wild animals in a natural reserve in Rajasthan State. The blood and lymph smears revealed organisms in spotted deer and sambar but parasites were not found in tigers, leopards, hyaenas, nilgai, wild boars, jackals, gazelles and black bucks examined.

Elephant trypanosomosis was reported by Sinha et al. 2002, based on blood smear and clinical signs, but the smears collected from 11 elephants in the present study did not reveal the organisms.

### 5.1.2 Isolates obtained by mouse inoculation

During the present study, it was possible to obtain three trypanosome isolates viz., of dog, leopard and lion. Among the three isolates it was observed that the canine isolates were most virulent. The organisms appeared on 4th day and the mice died on the 5th day post inoculation. In the case of leopard isolates trypanosomes appeared on the 5th day post inoculation and gradually parasitaemia increased subsequently on the 6th and 7th day. Large number of trypanosomes were observed on the 8th day post inoculation followed by death of the mice. It was noticed that the lion isolate was the least virulent. It was also observed that organisms appeared between 7th and 11th day post inoculation in the blood smear. The mice survived up to 26 days, with peak parasitaemia observed from 14th day post inoculation. This finding was in accordance with Queiroz et al. (2000) who had observed the variability in virulence of *T.evansi* isolated from horse and dogs.

### 5.1.3 Detection for *T.evansi* organisms by Quantitative Buffy Coat (QBC) method.

Amato Neto et al. (1996) could detect a single *T.cruzi* organism in mice by the QBC method. Similarly, in the present study the QBC method was helpful for detection of *T.evansi* organisms especially of lion isolates which were maintained in mice. Sometimes both wet and blood smear could not reveal the
organisms even after 7th day post inoculation in mice. By this method it was able to detect the organisms on 6th day post inoculation in the buffy coat region.

5.1.4 Passive haemagglutination test (PHA)

5.1.4.1 Antigen preparation

The first requirement for serological diagnosis of *T.evansi* is the need for isolation of host cell free parasites. This was accomplished by ion exchange chromatography (DEAE- cellulose) based on the principle differences in the surface charges. The negatively charged blood cells adsorb on the column, while the less negatively charged trypanosomes are eluted out (Lanham and Godfrey, 1970). The method is modified to obtain parasite free from host erythrocytes. The DEAE cellulose could separate trypanosomes from 95% of erythrocytes by differential centrifugation at low speed with repeated use of phosphate saline glucose buffer. This helped to avoid contamination of erythrocytes in the elute and 7 time greater volume of blood could be loaded to the DEAE cellulose column. Similarly Rosen *et al.* (1979) also followed differential centrifugation but used lactated ringer’s solution.

In the present study, PHA test employing soluble antigen of *T.evansi* parasite of canine isolate harvested from rats showed promising results.

. Gill (1970) observed that the homogenate prepared by disrupting the trypanosomes with ultrasonic vibrations proved to be the best antigen. Next in the order of efficacy was acetone dried organisms followed by lyophilized whole organisms and glycerinated suspension of the trypanosomes.

5.1.4.2 PHA test

Passive haemagglutination test has been adopted as a method of choice for the diagnosis of trypanosome infection by number of workers in cattle and buffaloo (Verma and Gautam 1977, Krishnappa *et al.*, 2002), camels (Jatkar and Singh (1970), goats ( Shen, 1974) and sheep (Saseendranath *et al.* 1995). Since reports are not available on PHA test in wild animals, the supportive guidelines have been taken from the earlier workers (Saseendranath *et al.* 1995, Krishnappa *et al.*, 2002).

Out of 113 samples screened, 64 (56.63%) serum samples were positive by PHA, the positive titres ranged from 1:32 to 1:512. A total of three known negative leopard cub serum was used as negative control.
which did not show any antibody titre in PHA. The titre of 1:32 was taken as cut off value to declare as positive. (Jatkar and Singh, 1971; Verma and Gautam, 1977; Raina et al. 1985 and Saseendranath et al. 1995).

The highest titre recorded in the present study was 1:512 in 4 lions and 3 tigers of Bannerghatta National Park and 1 tiger belonging to Mysore zoo (Table 3). However, high PHA titres of (1:1024) were reported in bovines by Verma and Gautam (1977) and 1:2048 by Krishnappa et al. 2002. Two animals from the Lion–Tiger safari at Thavarekoppa showed titre of 1:32 in the present study.

The observations from animals at the Shri Jayachamarajendra zoological park at Mysore revealed that out of 6 leopards screened only 1 showed titre of 1:64 and other 5 did not show any antibody titre, 2 Jaguars showed titre of 1:64 and 1:128 and one tiger indicated a titre of 1:512. The other 4 tigers which included a recently captured animal from the forest did not show indicative antibody titre. The screening of animals by PHA in bovines by Krishnappa et al. (2002), indicated that 27.58 % were positive in one of the organized farm in Mysore district. Mysore zoo has surrounding forest area and also irrigated agricultural lands which is suitable for the vector population. The prevalence of trypanosomosis in the present study based on PHA was less in Mysore compared with the other 2 zoological parks. This might be due to good managemental practice such as timely prophylactic dosing for trypanosomosis, vector control, good nutrition, regular deworming and one of the important factor is proper meat inspection and feeding of meat 6 hours after slaughter so the trypanosomes, if present, undergo disintegration (Murali Manohar et al., 2003).

The highest prevalence of *Trypanosoma evansi* have been recorded from lion-tiger safari of Thyavarekoppa, where, out of 6 leopards, 5 were positive (83.33%) in which 4 of them had a titre of 1:32 and one with 1:128. There were only two lions both showing antibody titre at 1:32. The leopards and lions were in the carrier state which was proved by getting isolates from these animals. Out of 11 tiger samples collected 7 showed positive (63.63 %), where 5 of them showed titre of 1:32, 1 showed 1:64 and other showed higher antibody titre at 1:128. The highest prevalence of trypanosomosis of 47.05 % in bovines has been reported based on PHA in Shimoga district when compared to other districts of Karnataka by Krishnappa et al., (2002). Since, this safari is located in a highly endemic area, regular prophylactic measures and utmost care should be taken for meat inspection and feeding since it is proved that carnivorism is one of
the mode of infection (Silva et al., 2007). It is a very difficult task for vector control because of location of the zoo in endemic area.

Out of 11 serum samples collected from elephants at Sakkarebyalu elephant camp, three (27.27%) samples were found positive by PHA. The antibody titre was 1:32, 1:64 and 1:128. The serum samples of 6 animals showed antibody titre of 1:16, which was considered to be negative. Renukaprasad et al. (1991) reported the incidence of Trypanosoma evansi in five captive tigers of the Bannerghatta National Park based on blood smear examination. In the present study, out of 43 lions belonging to rescue centre of Bannerghatta, 30 (69.76%) were found to be positive in PHA. Out of 14 tigers, 9 (64.28%) were found positive. All the three leopard samples examined were positive and these leopards had not been treated. The prevalence of Trypanosoma evansi in bovines by PHA in Bangalore Urban was reported to be 18.75% by Krishnappa et al. (2002). Out of 11 camels screened, 1 (9.1%) indicated the antibody titre of 1:32. Jatkar and Singh (1971) standardized PHA and considered antibody titres of 1:40 and above to be positive. Shahardar et al. (2004) from Surra endemic areas of Rajasthan in and around Bikaner reported that the double immunodiffusion test and counter immunoelectrophoresis detected 2% and 19.62% camels to be positive for T.evansi, respectively.

Variations in percentage of prevalence of T.evansi infection in different zoological parks of Karnataka may be attributed to vector population (Patchimasiri et al.,1983). Favorable habitats for tabanid flies such as forest lands, irrigation channels and temperate climate etc could be seen in parts of Karnataka which included Shimoga, Mysore and Bangalore Urban (Krishnappa et al., 2002). Carnivorism (Silva et al., 2007) may be one of the factors which attributes to the high percent of prevalence of T.evansi in Tiger-lion safari at Thyavarekoppa. In bovines, the highest prevalence of 47.05% in Shimoga district based on PHA was reported by Krishnappa et al., 2002, indicating it to be endemic for trypanasomosis.

5.2 Detection of Trypanasoma evansi by PCR method

Molecular diagnostic techniques particularly PCR is a promising technique for the diagnosis of trypanosome infection which is based on the detection of specific DNA sequences of trypanosomes. PCR technique uses thermo stable DNA polymerase and specific oligonucleotide primers to conduct repeated cycles of DNA invitro on a small amount of template DNA. Using this technique, a specific segment of the
trypanosome DNA can be amplified over a million times which makes the subsequent detection of that specific segment much easier. Omanwar et al. (1999) had reported that PCR amplification of DNA using parasite specific primers represented a potentially powerful tool for epidemiological studies in animal trypanosomosis.

Generally the disease surra is chronic in nature, very often the recovered animals exhibit low levels of fluctuating parasitaemia for years and serve as carriers for the disease. Several PCR methods have been developed for diagnosis, targeting different genes including kinetoplast DNA (Donelsen and Artama, 1998), repetitive sequence DNA (Artam et al., 1992 and Wuyts et al., 1995), ribosomal DNA (Ijaz et al., 1998) and internal mascribed spacer 1 (ITS-1) region og r-RNA (Taylor et al., 2008). However, PCR test targeting VSG gene is most reliable one so far diagnosis is concerned as the VSG is expressed in early, middle and late stage of the infection (Lin et al., 1985; Robinson et al., 1999). Hence it is possible to detect the infection in all stages. Moreover a major portion of the population surveyed was negative to blood smear/clinical examination for surra. Hence the selection of targeting VSG gene is quite appropriate in this study to detect the carrier status.

The polymerase chain reaction has emerged as a rapid diagnostic technique for many diseases including parasitic infections. PCR amplification of T.evansi specific DNA sequence is reported to detect the DNA equivalent of a single trypanosome (Viseshkul and Panyim, 1990 and Panyim et al., 1992).

In the present study the PCR based amplification was employed for identification of isolates of T.evansi derived from canine, leopard and lion. The PCR method was used in the study to detect T.evansi infection in captive wild animals which included leopards, jaguars, tigers, lions, elephants and also camels. In the present study, the primer pair (Urakawa et al., 2001) that yielded 488 bp amplicon which was also used by Ngaira et al. (2004) for detection of cameline trypanosomosis in Kenya and also Shailaja et al. (2005) for diagnosis of trypanosomosis in captive lions and tigers.

In the present study, DNA was extracted from blood of mice showing parasitaemia which were inoculated with canine, leopard and lion isolates. On analysis of PCR products by electrophoresis on 1.6% gel, amplicon of 488 bp was observed. These DNA samples were used as positive PCR controls for further epidemiological study of trypanosomosis in captive wild animals.
The DNA extracted from blood samples of leopards and lions belonging to Thyavarekoppa safari which yielded isolate, was used to detect *T.evansi* by PCR. However the PCR did not show generation of 488 bp amplicon. Similarly, the DNA sample from tigers of the Thvavarekoppa safari and from Mysore zoo did not show amplification at 488 bp. These findings were not in accordance with Shailaja *et al*. (2005) where they reported amplification at 488 bp in samples from tigers and lions.

In the DNA extracted from frozen blood samples of eleven elephants of Sakkarebyalu camp, amplicon of 488 bp was found in five samples. Out of 11 DNA samples of camels, nine showed the DNA amplification at 488 bp. This result was in accordance with findings of Ngaira *et al*. (2004).

It was surprising to note that isolates from wild animals including leopard and lion yielded amplicon of 488 bp, where as DNA extracted from blood from respective wild animals did not show amplicon at 488 bp. The DNA sample extracted from frozen blood samples of elephants yielded amplicon at 488 bp and similar result was obtained from camels. These results are in accordance with Ngaira *et al*. (2004) and Shailaja *et al*. (2005), which suggested amplification of RoTat 1.2 VSG gene by PCR in *T.evansi*. Pacheco *et al*. (2005) observed that randomly amplified polymorphic DNA (RAPD) analyses and isoenzyme profiles were required for better results and therefore examination of more samples from different locations would throw more light. Shahardar *et al*. (2009) suggested further studies on the DNA fidelity of the segments co-amplified by hybrization technique using speicies-specific DNA probe to require overcoming nonspecific amplimers which may occur due to cross amplification of DNA sequences from host DNA complementary to the targeted sequences or possible contamination of these blood samples processed for PCR.

There is biometrical alteration of *T.evansi* isolates when there is change from horse to rodents which was observed by Davila *et al*. (1998). Genetically, there may be change in the gene sequence. Attempt was made for gene sequencing of PCR product of leopard, lion and tiger but the sequence did not match with each other.

**5.3. Comparision of *T.evansi* isolates**

In the present study, the partial sequence alignment report and phylogenetic tree of VSG gene nucleotide sequence of 3 isolates and the reference strain (NO. AF 317914) showed that similarity and diversity ranged from 92 to 99.6 and 0.4 to 3.5 percent respectively.
The phylogenetic tree indicated that the canine and lion isolates belonged to different clusters and there is wide percentage identity of 92 and 3.2 percent with regard to divergence. The leopard isolate and reference were different and belonged to the cluster of canine isolate. The percent of identity of canine isolate and that of leopard was 98.6 and divergence was 0.5. However, a similarity co-efficient of 40 to 100 percent was observed between isolates of *T.evansi* from dog and camel. Ngaira *et al.* (2004) identified different *T.evansi* strains of camels in Kenya based on the absence of RoTat 1.2 VSG gene by PCR and confirmed by isoenzyme profile. Multilocus enzyme electrophoresis (MLEE) and RAPD analyses were used to investigate the genetic heterogeneity in 18 *T.cruzi* strains isolates (Pacheco *et al.*, 2005).

This is the first report where the partial gene sequence of leopard and lion isolates was attempted. In the present study however heterogeneity in virulence and genetic divergence was found between isolates.

This epidemiological study helped to know the heterogeneity in the virulence of isolates of *T.evansi* in mice. It was observed that canine isolates were highly virulent followed by the leopard isolates. Based on the phylogenetic tree it was proved that both belonged to the same cluster. In contrast, it was observed that the lion isolate was less virulent and belonged to a different cluster. In the present study the observation noticed in the virulence of different isolates helped to correlate with the help of phylogenetic tree.

Earlier workers have proved, by PCR that diagnosis was possible even in the presence of a single *T.evansi* organism. In elephants and camels, PCR method was able to detect the latent infections which were confirmed by appearance of the amplicon of 488 bp. The blood smears and mouse inoculation test did not yield results, whereas the passive haemagglutination test confirmed the presence of antibodies. Although isolates maintained in mice and rats showed specific 488 bp amplicon, PCR did not show presence of 488 bp amplicon with blood samples of lion, leopard and tigers when directly subjected to PCR. Perhaps it could be due to presence of some PCR inhibitory factors in blood samples of wild animals.

The present investigation concludes that the host factors and the genetic level adaptability of *T.evansi* organisms in lab animals should be taken into consideration for analysis of epidemiology in trypanasomosis. The PCR inhibitory factors in blood samples of lion, tiger and leopard need through investigation, so that this technique can be used effectively in the confirmatory diagnosis of *T.evansi* infection in these wild species.