CHAPTER – 5

DISCUSSION
5. DISCUSSION

In domestic livestock, surra/trypanosomosis is caused by the range of trypanosomes such as *T. evansi*, *T. brucei* and *T. equiperdum* leading to economical loss in animal husbandry industry. Several diagnostic tests and its subsequent treatment were explored as the effective control regime and to reduce the carrier status of the animals. Several diagnostic tests were used by several groups for the diagnosis of surra such as nucleic acid detection by PCR (Sengupta *et al.*, 2010; Rudramurthy *et al.*, 2013), microscopic examination, parasitological and serological tests. Earlier studies showed that microscopic examination failed to detect more than ~50% of *T. evansi* infection (Pathak *et al.*, 1997). Moreover, acute and sub-acute infections can also be satisfactorily diagnosed by conventional parasitological techniques but when the parasitemia is very low, it becomes very difficult to detect the latent or chronic infection. Further, by parasitological diagnostic tests only clinical stages of infections can be diagnosed satisfactorily but not the latent or chronic infection (Fernandez *et al.*, 2009). Thus, in such scenario, serological tests are having more advantage for the detection of carrier status of animals as the animals once infected may harbour the infection for long time if remained untreated (Atarhouch *et al.*, 2003) and such tests are applicable for mass screening in the field for effective control of the disease with better production (Lejon *et al.*, 2003). The serological test such as ELISA qualifies as a universal test for correctly identifying healthy animals as it is not strain specific (OIE 2012). However, the efficiency of sero diagnosis of the test was depended on the selection of potent antigens.

Sero prevalence ELISA assay based on *T. evansi* crude surface antigens were reported earlier by many workers from different parts of the world (Kundu *et al.*, 2013). *T. evansi* WCL antigen and antibovine conjugate has proved to have adequate qualities in sero diagnosis in buffaloes (Kocher *et al.*, 2015). It was reported earlier that immunoblotting characterization of different stocks of *T. evansi* by antibody of various host species helps in understanding of the host-parasite relationship, pathogenicity and immunogenicity of *T. evansi* (Aquino *et al.*, 2010). In appearance, *T. evansi* is longitudinal and fusiform and its flagellum protrudes from the basal body. The parasites are attached to the host surfaces by flagellum (Bastin *et al.*, 2000). For the possible immunological intervention, the flagellar pocket region can be considered as the prevediled site (Radwanska *et al.*, 2000) and its associated proteins have been identified and found to contribute to trafficking and virulence (Field and Carrington, 2009). Moreover it has also been reported that
paraflagellar rod protein may be the critical organelle mediating attachment to vector cell surface (Gadelha et al., 2005) and are involved in the binding of macro molecule interactions with the host and elicit host antibody response. Further, paraflagellar rod protein gene 1 (PFR 1) and paraflagellar rod protein gene 2 (PFR 2) have been cloned and expressed in prokaryotic system (Maharana et al., 2011a; 2011b). Thus, for the development of vaccine and diagnostic test, flagella pocket antigens and paraflagellar rod protein has been explored (Mkunza et al., 1995; Obishakin et al., 2014). Earlier studies have also shown that, FLA portion of T. evansi mediates host parasite interactions and immunogenicity (Langousis et al., 2014; Singh et al., 1995). SDS-PAGE analysis of FLA antigen of T. evansi showed nine bands ranging from 18-130 kDa. It was also mentioned in the earlier studies that even in some geographical regions, 11 major peptide bands were found in the range of 33 to 81 kDa for native whole cell lysate antigen followed by 4 to 12 polypeptide bands found in the range of 17.6 to 80.2 kDa for FLA antigen and also the polypeptide bands were varied in different isolate (Singh et al., 1995). The FLA antigen remained non-reactive in I-ELISA with T. annulata and B. bigemina infected sera. Moreover, FLA antigen of T. evansi showed immunoreactivity in ELISA and SDS analysis with experimentally produced T. evansi hyperimmune sera raised in rabbit/rat and with the panel of sera samples consisting of experimentally produced field samples including different host species namely cattle, buffalo, camel, horse and donkey. On comparison with CATT/T. evansi test, the developed FLA-I-ELISA test showed a potent diagnostic performance of 98.1% sensitivity, 97.4% specificity and agreement value of Cohen’s kappa coefficient of 0.89. The statistical analysis of species and age-wise epidemiological data showed no significant variations were observed when compared between two test system i.e. I-ELISA and CATT/T. evansi (χ²=0.14, df=1, p>0.05). The sero prevalence study revealed that the adult cattle (up to 21%, χ²=0.06, df=1, p> 0.05) showed more disease prevalence compared to calves and opposite was observed among buffaloes where calves were showing more incidence of T. evansi infection compared to adults (Table 5). However the disease prevalence in horse, donkey and camels were found to be equivocal among different age groups (P> 0.05). Thus, the comparative analysis of FLA antigen based I-ELISA test were comparable with CATT/T. evansi. Further, the present study provides the preliminary information of T. evansi antigens to be explored in I-ELISA for the detection of surra based on age groups of different species with the limited number of serum samples. Thus, in field conditions with high percentage of chronically infected animals, FLA-I-ELISA using FLA antigen of T. evansi can be
definitely explored as a diagnostic tool in terms of good sensitivity and specificity for the effective control regime for mass screening of antibodies of *T. evansi*.

5.1. CI-ELISA

FLA antigen based CI-ELISA was developed exploring MAbs (FLA-MAbs). The developed FLA-MAbs showed high immunoreactivity in immunoblot analysis and ELISA respectively. FLA-MAbs belongs to isotype IgG with IgG_{3} subtype. Earlier MAbs were produced by several groups against different antigens of trypanosomes such as against metacyclic trypamastigotes stage of *T. cruzi* (Araujo *et al.*, 1982, Manas *et al.*, 1986), invariant antigens of vector form of *Trypanosoma simiae* (Bosompem *et al.*, 1995) and associated antigens against *T. cruzi* (Takasu *et al.*, 1989). It was found that, the MAb based ELISA (CI-ELISA) have significance importance in the diagnosis of parasitic diseases and showed high specificity as the antibodies were produced against single epitope and were found to be organism specific. Further, any serum from any animal species can be tested in CI-ELISA by using single anti-mouse immunoglobulin conjugate in less time with low expenditure. CI-ELISA remained reactive with experimentally produced rat/ rabbit hyperimmune sera and with field sera samples. Moreover, it remained non-reactive with control sera samples and with sera samples clinically infected with *T. annulata* and *B. bigemina*. Further, the developed test also detected antibody from different host species such as cattle, buffalo, horse, donkey and camel.

The statistical analysis showed no significant variations between the two test systems i.e. CI-ELISA and CATT/*T. evansi* ($\chi^{2}=0.002$, df=1, p>0.05). Moreover, FLA antigen based CI-ELISA test showed optimum combination of sensitivity and specificity of 95.8 and 94.4, respectively. The positive-negative cut off percentage inhibition (PI) value was found to be >55, with a Cohen’s Kappa value of 0.83. Further, no significant variations were observed among different species of two tests (p > 0.05). State-wise sero-prevalence studies also showed no significant variations among two tests (p> 0.05). Thus, the developed CI-ELISA based on FLA antigen and its related monoclonal antibody can be explored as an excellent diagnostic tool for the detection of antibody against *T. evansi*. However, the cross-reactivity of CI-ELISA with the antibody of other flagellated protozoa needs to be explored further. As per the literature search, this is first ever endeavor to explore monoclonal antibody produced against FLA antigen of *T. evansi* for the development of CI-ELISA for the sero diagnosis of surra/trypanosomosis.
5.2. Ag-ELISA

After treatment the trypanosomal antigens were cleared within 2 weeks (Nantulya and Lindqvist, 1989). Thus the rapid disappearance of antigen in animals after treatment from the circulation confirms successful treatment and also minimizes false positive results. Even though antigen level decreases after treatment, antibody level continues to increase. Such diagnostic test should be implemented which is directly capable of detecting antigen in the circulation, thus minimizing false positive results. In cattle and camel, Ag-ELISA was shown to have high diagnostic sensitivity of more than 90% and 95% respectively (Nantulya et al., 1989; Nantulya 1990). Further, in experimental infected goats and cattle for over 2 years of period with *T. congolense*, could detect antigens at 94% and 82% respectively (Masake and Nantulya, 1991). In the present study, double antibody sandwich ELISA (Ag-ELISA) exploring FLA antigen of *T. evansi* and its related MAbs were developed to detect the circulating trypanosomal antigens.

The blood samples collected from cattle clinically infected with *B. bigemina* and *T. annulata* and control blood samples remained non-reactive in Ag-ELISA. Further, *T. evansi* antigen were detected in Ag-ELISA when subjected to field blood samples collected from cattle, buffalo, horse and camel. The diagnostic PCR assay amplified the genomic DNA of 400 bp and was used as reference test against Ag-ELISA.

The statistical analysis of Ag-ELISA using ROC analysis showed 88.2% sensitivity, 86.8% specificity, cut off O.D value > 0.33 and Cohen’s kappa value of 0.84 when compared with the standard diagnostic PCR assay as reference test. Moreover, seroprevalence study using 701 blood samples of different species including cattle, buffaloes, horse and camel from different states of India showed no significant variations in both the test systems i.e. Ag-ELISA and PCR (p>0.05) and both the tests were found to be in agreement. However, the evaluation of sensitivity by the dilution method using *T. evansi* infected rat blood, plasma and purified samples showed that, the Ag-ELISA detected 33.26 trypanosomes/ml from blood, plasma and purified sample. Thus, the present finding proved that Ag-ELISA exploring MAbs can be used as an rapid, excellent, reliable and promising diagnostic tool for the detection of trypanosomal antigen for mass screening in the field for sero surveillance of ‘surra’ in animals.
5.3. Epidemiology

Surra/trypanosomosis in animals were caused by *T. evansi* infection and were prevalent in many tropical countries, including India. Trypanosomosis decreases productivity in animals and causes severe loss in animal husbandry industry as many animals acts as carrier for *T. evansi* infection. Thus it is necessary to diagnose and treat carrier animals for complete stamping out the disease as control regime. Sero-prevalence studies by the developed diagnosed assay using FLA antigen of *T. evansi* and MAbs with field sera samples collected from different species of animals showed that the disease were more prevalent in buffalo, cattle and camel. However, very less sero-prevalence of trypanosomosis was observed in donkey and horse with 6.89% and 5.10%. The present findings showed an overall approximately 24% sero-positivity in animals of all the tests which were comparable to the earlier reports of 40% in eastern India (Laha and Sasmal, 2009). Further, no significant variations were observed when compared with reference tests within each species in each state. (Table 5, table 6 and table 7). Thus, the developed tests were in agreement with standard tests (p> 0.05). However, larger panel of sera samples from different species and from different states of India need to be evaluated to provide reliable data for the complete sero-prevalence study of surra/trypanosomosis.