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Clinical Features of Chronic Trypanosomosis in Rabbits Experimentally Infected with the Malaysian Isolate of*Trypanosoma evansi*

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Abstract: *Chronic trypanosomosis caused by Trypanosoma evansi is still less studied specially with regards to clinical features and this formed the basis of this study. A total of 35 adult male rabbits were divided randomly viz; G1, G2, G3, G4, G5, G6 and a control comprising of 5 animals each, based on the duration of monthly exposure. Trypanosome-infected rabbits were inoculated intravenously with 10^5 trypanosomes/ml while the control group received phosphate saline glucose (PSG) via the same route. The protozoan was found in the blood 72 hours,post infection (p.i). All infected rabbits showed different clinical signs which include fever, anorexia, loss of condition, emaciation, pale mucous membrane of eyes, oedema of testicles and in tissues around the anus, blepharitis, oedema of the face and corneal opacities. A significance decrease ($P<0.0001$) was seen in the body weights of the infected rabbits. The mortality rate was 16.67% (5/30). There was a gradual elevation of rectal temperaturefollowing the development of the parasitemia with a weak but significant inverse correlation ($r =- 0.124$; $p<0.001$). Moreover, cytokines such as TNF-α,INF-γ, IL-6, and IL-10 were measured and compared with the level of body weight, rectal temperature and anaemia. Our findings indicated that the clinical markers of Malaysian isolate of T. evansi in chronically infected laboratory animal model was included body weight changes, anaemia, pyrexia, oedema and orchitis and rabbit appears to be a suitable model in studying of T. evansi infection.*

Keywords:*Trypanosoma evansi, clinical features, cytokines, rabbits, chronic trypanosomosis*

I. Introduction

Trypanosoma evansi is a protozoan known as a causative agent of a wasting disease named surra [1]. It is mechanically transmitted via blood sucking flies such as those of Tabanus and Stomoxys[2].However, the infection is not only limited to bovine but spills over to equine, canine, feline, lapine and large murine (capybaras) and humans [3, 4].Ithas a widespread geographical distribution among the other trypanosomes species infecting livestock around the world and causing severe symptoms in horses and camels associated with a high mortality rate, whereas the signs are milder in other animal species [2]. The severity and outcome of the disease depends on the virulence of the strain, species of the affected host, duration of infection and the endemicity of the region [2]. Donkeys experimentally infected with the Brazilian strain of T. evansi survived up to 145 days p.i. but that infected with an Indian strain only survived up to 42 days p.i. [5]. Furthermore, the Mindanao strain of T. evansi from the Philippines is much more virulent that that of the Indonesian strain [6]. Recently, high mortality rate has been observed in goats, buffaloes and cattle, this is suggestive of the emergence of highly virulent strains [7]. In the past decades, severe outbreaks of surra have been reported which was associated with high incidence of anti- trypanosomes drugs resistant strains [8]. In Malaysia T. evansi was isolated from deer, cattle, buffaloes, pigs and horses[9, 10].

T. evansi causes fever, anaemia, weakness, oedema, losses in meat and milk production, reduced fertility, and sometimes abortion and death in the absence of treatment in different animal species (11, 2]. Anaemia is an important and predominant feature in animals infected with trypanosomosis, measured by decreased in PCV, RBC and Hb levels as pointers of erythrocytes response[5,12]. In trypanosomosis, interferon-gamma (IFN-γ) stimulates macrophages to induce the cytokines production such as TNF-α, IL-6 and IL-10 that plays a significant role in the replication process of the parasite and in the immune response of the host [13].

The rabbit is more suitable than the mouse or rat, not just because of the ease with which blood samples can be gotten, but also because the rabbit mimic the actual host does not exhibit high parasitaemia observed in rodents. In rabbits, trypanosomosis has a chronic course, characterized by apathy, pale mucous membranes and oedema of the eyelids and ears with fluctuating peaks of parasitaemia for protracted periods, providing the basis for rabbits being suitable models for study of the disease[14]. Apart from this obvious advantage, rabbits are easy to handle as an experimental model for the study of trypanosomosis which will permit the investigation into the pathogenesis, the diagnosis, prevention and treatments with the utmost goal of direct application in the field [15]. However, of the widespread distribution and host range of T. evansi, it still

remains the least researched in comparison to tsetse-transmitted trypanosomes. There were also no studies on the clinical signs for a protracted period of six months caused by Malaysian isolate of T. evansi in rabbits as laboratory animal models. Furthermore, there is no data concerning the levels of cytokinessuch as TNF-a, IFN-γ, IL-6 and IL-10 in chronic trypanosomosis. Therefore, this study aims to evaluate the clinical signs of rabbits experimentally infected with Malaysian isolate of T. evansi and providesinformationregarding the levelsof these inflammatory cytokines in T. evansiinfected rabbits and to establishtheirrelationshipwith clinical features of the disease.

II. Materials And Methods

2.1 Ethical approval

The study was approved by the Animal Care and Use Committee (ACUC), Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM/FPV/PS/3.2.1.551/AUP-R159).

2.2Experimental animals

A total of 35 adult male New Zealand White rabbits weighing between 2–3 kg were purchased from local supplier (RK Northern Supplies, Malaysia). Animals were placed within individual cages in a fly-proof wire netted room with temperature ranging from 23–28°C. They were acclimatised for 14 days where a commercial diet (Gold Coin, Malaysia) and water was provided ad libitum. Haematological and biochemical examinations were performed twice during this period to ensure that the rabbits are free from trypanosome and werein perfect health. During this period, the rabbits were dewormed by subcutaneous injection of Biomectin.

2.3Experimental Design

Five rabbits which acted as the controls were intravenously injected with 1.0 ml of PSG. The remaining 30 rabbits were also intravenously injected with 1.0 ml of the blood containing $1x10^5$ trypanosomes/ml *T. evansi* isolated from bovine in 2007 (Cattle isolate, Te002 from Johor-south) in Peninsular Malaysia and kept in liquid nitrogen and designated as follows, viz infected for 1, 2, 3, 4, 5 and 6 months p.i. were coined as G1, G2, G3, G4, G5 and G6, respectively. Rabbits from G1-G6 were all killed and necropsied at the end of the stipulated period while that of control group was killed after 6 months.

2.4Clinical assessment

The weights of the rabbits in each group were measured at intervals of 10 days using sensitive weighing balance before the collection of the blood. This procedure was carried out gently in order not to excite the animal.

2.5Body temperature

The body temperature was measured using a well-lubricated thermometer.

2.6Parasitaemia Evaluation

After the animals were inoculated with *T. evansi*, the level of parasitaemia was determined twice weekly by the collecting blood through the central ear artery using micro-hematocrit centrifugation technique (HCT) [16] and by hemocytometer [17] when the parasitaemia is high.

2.7Haematology

Complete haemogram was performed immediately after blood collection. The parameters include PCV, total RBC and Hb concentration were measured using an automated blood cell counter (Abbott Cell DYN 1700, USA).

2.8 Cytokines

Serum cytokines TNF-α, IFN-γ, IL-6 and IL-10 concentrations were determined using a commercial available kit (CUSABIO, BIOTECH, China) following the manufacturer's instruction. Briefly, 100 µl of rabbit's serum and TNF-α, IFN-γ, IL-6 and IL-10 standards in serial concentrations (5000, 2500, 1250, 625, 312.5, 156.25, 78.13, 0), (4000, 2000, 1000, 500, 250, 125, 62.5, 0), (1000, 500, 250, 125, 62.5, 31.2, 15.6, 0) and (2000, 1000, 500, 250, 125, 62.5, 31.2, 0) pg/ml respectively were added in a duplicate to each well coated with TNF-α, IFN-γ, IL-6 and IL-10 specific antibodies, then incubated for 2 hours at 37 ºC. 100 µl of biotin labeled with TNF-α, IFN-γ, IL-6 and IL-10specific antibody conjugates was addedafter serum removed, then incubated for 1 hour at 37 ºC.

HRP-avidin (100 µl) was added to each well afterwashingthem three times then incubate for 1 hour at 37 ºC. TMB substrate (90 µl) was added to each well after washed and incubated for 25 minutes at 37 ºC. In the

last step, stop solution (50 µl) added to each well, and the optical density determined by ELISA reader set to 450 nm within 5 minutes. Professional curveExert 1.4 was used to plot a standard curve.

2.9Statistical analysis

The data were statistically analyzed by one-way analysis of variance (ANOVA) and the means were compared using Tukey's test and expressed as mean±standard deviation (SD) which was analysed using JMP 9 (SAS, QSAS Institute Inc, Cary, NC, USA). Values were considered significant at $p<0.05$.Moreover,parasitaemia values were log-transformed for normalization and graph performance of the data.Pearson's correlation coefficient (r) and linear regression analysis (R^2) were performed on the data.

III. Results

3.1 Clinical manifestations of the rabbits

All infected rabbits showed different clinical signs which include fever, anorexia, loss of condition, emaciation, pale mucous membrane of the eyes, oedema of testicles and in tissues around the anus, blepharitis, oedema of the face and corneal opacities (FigIa-g). Table I shows the clinical outcome of rabbits during the experimental period.

Table II show the body weight of rabbits from all groups during the experimental period. A significant difference ($P<0.0001$) was seen in the body weights of rabbits at G1, G2, G3, G4, G5 and G6 compared to the controls. While, the control showed a 23% increase in body weight, those G1-G6 collectively lose 6.4%, 7.2%, 8.5%, 7.4%, 7.5% and 2.2% at one, two, three, four, five and six months p.i. respectively.

The time of death and hematological parameters of affected rabbits are presented in Table III.The morbidity rate noticed in the infected groups was 100% (30/30), while the mortality rate was 16.67% (5/30). The first animal from G3 died, whereas the other four rabbits were euthanized due to the poor body condition.

3.2 Hematological findings

FigII shows the haemograms of selected blood parameters in the animals during the experimental period. Rabbits in the infected groups have significantly lower ($P<0.0001$) RBC counts, Hb concentrations and PCV values commencing from G1 until the end of the experiment.

3.3Parasitaemia and pyrexia

No parasite was detected by HCT in the control group during the study period. All infected rabbits developed parasitaemia on day 3 p.i. as detected by HCT while the first peak of mean parasite count per ml of blood was noticed on day 11 p.i. Similarly, the control group did not show evidence of pyrexia during the experimental period with mean rectal temperature of 38.5±0.17°C.

FigIII shows the mapping of parasitaemia against rectal temperature. The rectal temperature was elevated gradually following the development of the parasitaemia with a weak but significant correlation ($r = -0.124$; $p<0.001$). Nevertheless, an increase in rectal temperature is attained despite a decline in parasitaemia.

3.4 Cytokines

Fig IVshows the concentration of TNF-α, IFN-γ, L-6 and IL-10 in the serum of rabbits during the experimental period. The TNF-α, IFN-γ levels in the infected animals were significantly ($P<0.0001$) higher than that of the controls at G1-G2. Nevertheless, IL-6 was significantly ($P<0.001$) higher at G1-G2 compared to the control group. However, the concentration of IL-10 at G1-G2 was comparable to the control, but at G3-G6, the levels are higher ($P<0.0001$) than the control.

Fig V a, b, c and d show Pearson's correlation (r) and regression (R2) analysis of the paired data comparing the variables of INF-γ, TNF-α, IL-6 and IL-10 with body weight.A negative correlations were observed between INF-γ and body weight ($r=-0.38$, $R^2=0.144$, $P<0.04$), TNF-α with body weight ($r=-0.562$, $R^2=0.316$, $P<0.001$), IL-6 with body weight ($r=-0.548$, $R^2=0.3$, $P<0.002$) and IL-10 with body weight ($r=-0.426$, $R^2=0.181$, $P<0.002$).

Fig VIa and b show the Pearson's correlation (r) and regression (R^2) analysis of the paired data comparing the variables of TNF-α and PCV with body temperature. A positive correlation was found between TNF-α and body temperature ($r=0.584$, $R^2=0.341$, $P<0.001$). However, a negative correlation between body temperature and PCV ($r=-0.623$, $R^2=0.388$, $P<0.0001$) was observed.

Fig VIIa, b and c show Pearson's correlation (r) and regression (R^2) analysis of the paired data comparing the variables of TNF-α, IL-6 and IL-10 with PCV. A negative correlations were observed between TNF-α and PCV ($r=-0.372$, $R^2=0.139$, $P<0.05$), IL-6 with PCV ($r=-0.375$, $R^2=0.141$, $P<0.05$) and IL-10 with PCV ($r=-0.661$, $R^2=0.436$, $P<0.0001$).

IV. Discussion

The T. evansi used in this study caused clinical trypanosomosis in all infected rabbits, characterized by the reduction in body weight throughout the six months of the study period compared to the controls which incessantly gained an average weight of 23%. Similar findings of weight lost were also reported in rats infected with T. brucei[18], goats and sheep infected with T. congolense[19, 20]and goats infected with T. evansi, T. brucei and T. vivax[11, 21]. These findings along with that of the present study denote that infection by trypanosome can be considered that of a wasting disease.

The decrease in the body weight during trypanosomosis in the study reported here may be related to the anorexic most likely initiated via pro-inflammatory cytokines[22]. Furthermore, in the present study, this could also be associated with the deleterious effects of the cytokines IL-6, IFN-γ, 1L-10 and TNF-α release in the direct or indirect reduction of feed intake. Their effect on glucose-sensitive neurons of the hypothalamus and through the stimulation of the hypothalamic prostaglandin E2 synthesis, which in turn stimulates the release of corticotrophin releasing factor (CRF) from the hypothalamus[23, 22].

A puffy eyelid that was observed in the present study was associated with nephrotic syndrome related to the leakage of proteins through the kidney which probably can cause the oedematous appearance of the eyelids and "self-inflicted" blepharitis as a result of scratching of the eyelids.

Usually, the infection is characterized by high mortality in goats and cats infected with T. evansi amounting to more than 40%[11, 12]. However, in the current study, the mortality rate was 16.67%. The difference in the mortality rate may be related to the differences in animal species, number of animals that used in each experiment and parasitic strain virulence of the causal organism. Nevertheless, rabbit has been successfully used as models for vaccination against trypanosomosis[24] explaining its hardiness as seen in this study with a lower mortality following infection.

The emergence of parasitaemia also differs between species where in goats infected with $5x10^3$ and $5x10^4$ trypanosome/ml it appeared 2-7 days p.i. [11] and in cats infected with $1x10^8$ an earlier appearance is seen at 1-2 days p.i. [12]. In the current study, emergence of parasitaemia at 3 days p.i. is longer than that of cats [12]but earlier than those of goats [11]. The differences in the prepatency period may perchance be related to the differences in the dose size, strain virulence of T. evansi and animal species [11]. Nevertheless, in the present study, the waves of the parasitaemia followed by the initial detection of the parasite in the blood could probably be initiated to permit the parasite to evade the host's immune system via antigenic variation of VSG [25].

The rectal temperature of the infected rabbits rose steadily and coincided with the appearance of the parasite in the blood and peaked on day 25 p.i. which was consistently higher than the control group throughout the course of the infection. This increment and the appearance of the parasite in the blood in the present study are possibly associated with the parasite's toxic metabolites[26]. Apart from inciting haemolysis and a drop in PCV [27], these by-products play a significant role as pyrogens[22] stimulating fever.

The body temperature was positively correlated with TNF-α in the present study. The TNF-α produced by peripheral blood mononuclear cells (PBMCs) may be recognized as an indication of a pyrogenic signal by certain regulatory sites within the hypothalamus in the central nervous system [28]. Moreover, TNF-α induces prostaglandins synthesis, which represent the central mediator of the coordinate response which leads to fever[29]. Thereafter, this cytokine turns up the set point of the physiologic thermostat and caused the body temperature to increase[29].

Parasitaemia paralleled with the temperature, suggesting the association of the pyrogenic cytokines with the trypanosomes numbers in the circulation and also in the tissues [11, 30]. In the current study, the intermittent rise in rectal temperature rises even after a drop in the parasitaemia may be related to the continuous existence of pyrogenic cytokines[31, 29, 30]. This is in contrast to the previous reports with no correlation between temperature and parasitaemia in calves[32], sheep, capybaras and coati [33, 34, 35]. However, the findings in this study resembled those in goats and horses[19, 36]. As previously discussed, it is likely that the ability of rabbit immune system to mount a continuous response to the presence of trypanosome has led to such findings [24].

Anaemia is the main finding in all infected animals with T. evansi and become incessant after the decline in parasitemia, so there are other factors involved rather than the parasite such as inflammatory cytokines. Therefore, in the current study there was a decline inthe PCV which was attributed to the increase of IFN-γ, TNF-a andIL-10showing that the increased of the cytokines levels are associatedwith anemia. Furthermore, the elevation of these cytokines were associated with the immune system regulation against the parasite at the same time contributing to the development of anemia in rabbits infected with T. evansi. Moreover, pyrexia also has a relationship with anaemia as it increases the osmotic fragility which changes the permeability of erythrocytes membranes [37]. Additionally, there was a negative correlation between pyrexia and PCV as was observed in the present study.

V. Conclusion

The present study evaluates the clinical markers to assist in the diagnosis of T. evansi, including body weight, anaemia, pyrexia, oedema and death are suggestive of the deleterious effect of the Malaysian isolate of T. evansi in rabbits. In addition, orchitis was the prominent clinical sign in the rabbits which aided in the gross diagnosis of the infected animals as early as the second week p.i. It appears that this experimentally infection of chronic trypanosomosis develops into a multisystemic disease in rabbits.Furthermore, a synergism among INFγ, TNF-α, IL-6 and IL-10 contributing to the body weight losses, while TNF-α, IL-6 and IL-10 was connected withanaemia development and linked to the regulation of immune responses against theT. evansi.Additionally, therewas association between TNF-γ and PCV with body pyrexia. Last but not least, rabbit appears to be a suitable model in studying T. evansi as the mortality is low, allowing assessment into progression from acute to chronic stages.

Declaration of Conflicting Interests

Authors declared no conflicts of interest with respect the research and publication of this article.

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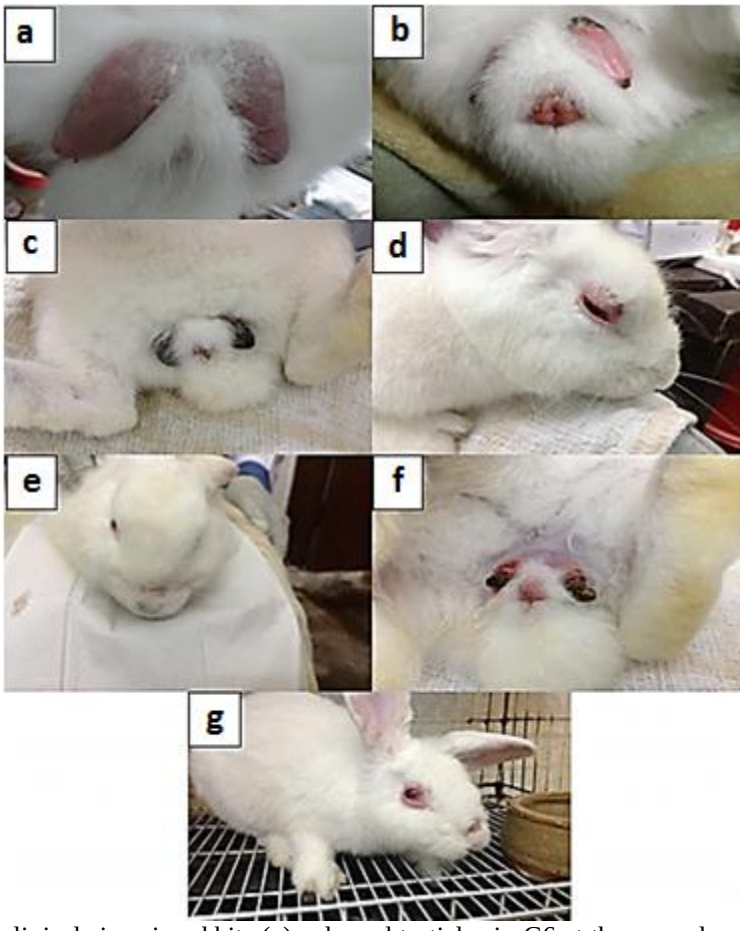


Fig.I: Photographs of clinical signs in rabbits (a) enlarged testicles in G6 at the second week p.i. (b) oedema of testicle and in the tissues around the anus with necrotic areas in the anterior and posterior parts of testicle in G2 at four weeks p.i. (c) shrunken testicles and necrotic scrotum of G3 at eight weeks p.i. (d) blepharitis in G2 at eight weeks p.i. (e) oedematous face in G4 at the ninth weeks p.i. with slight alopecia around the nasum (f) shrunken testicles with necrotizing tips and oedema of tissues around the anus in G5 at 18 weeks p.i. (g)buffy eye with corneal opacity and distinct alopeic area above the nose in G6 at 20th weeks p.i.

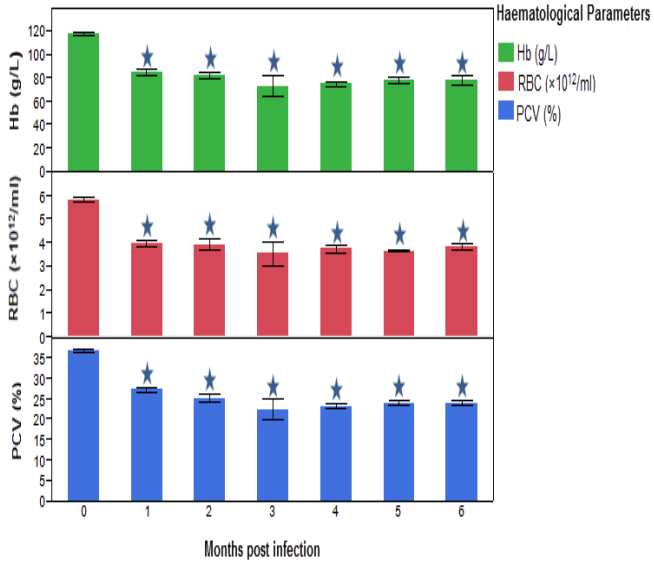


Fig.II: Hematological parameters PCV, RBC and Hb levels in control and *T. evansi* infected rabbits (refer to the changes between theinfected and control groups of rabbits are significant at ($P < 0.0001$)

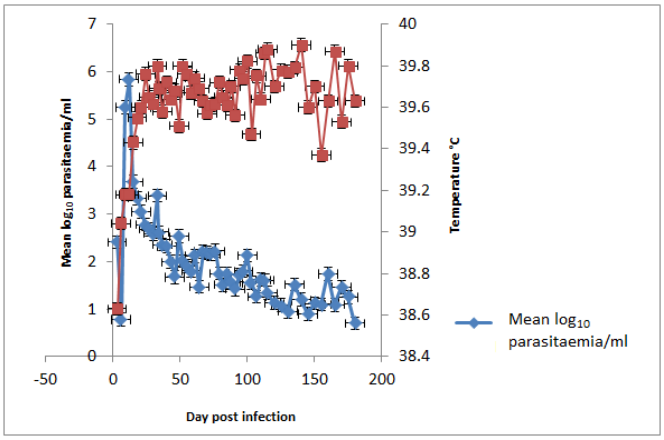


Fig. III: Mean $\log_{10}$ of parasitaemia and body temperature of *T. evansi*-infected rabbits during the six month period

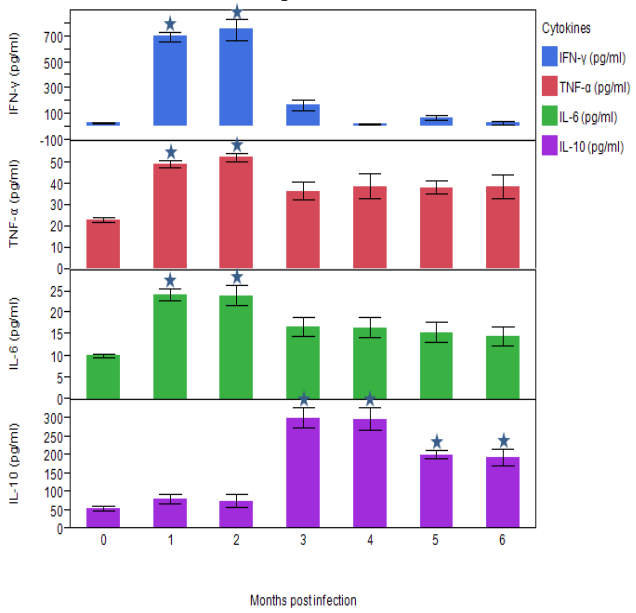


Fig. IV:IFN-γ, TNF-α,L-6 and IL-10 concentration of rabbits during the experimental period. Refer that changes between the infected and control groups are significant) (Error bar = 1 standard deviation)

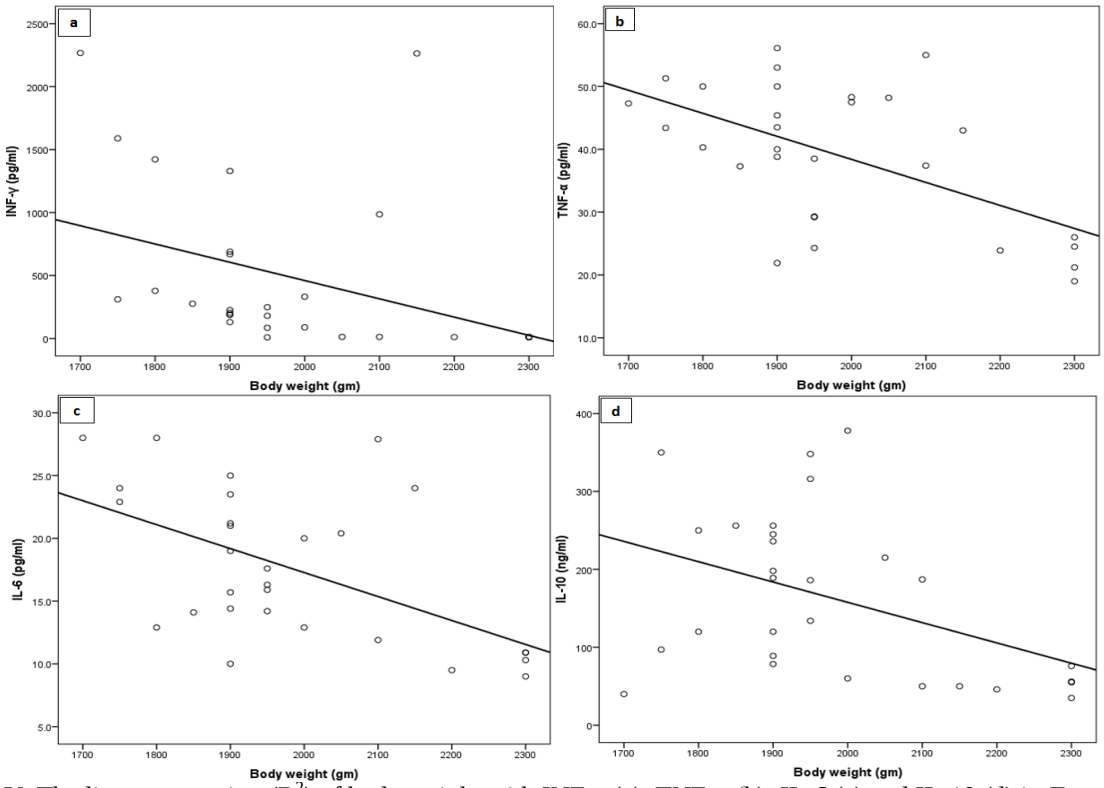


Fig. V: The linear regression (R^2) of body weight with INF-γ (a), TNF-α (b), IL-6 (c) and IL-10 (d) in *T. evansi* infected rabbits

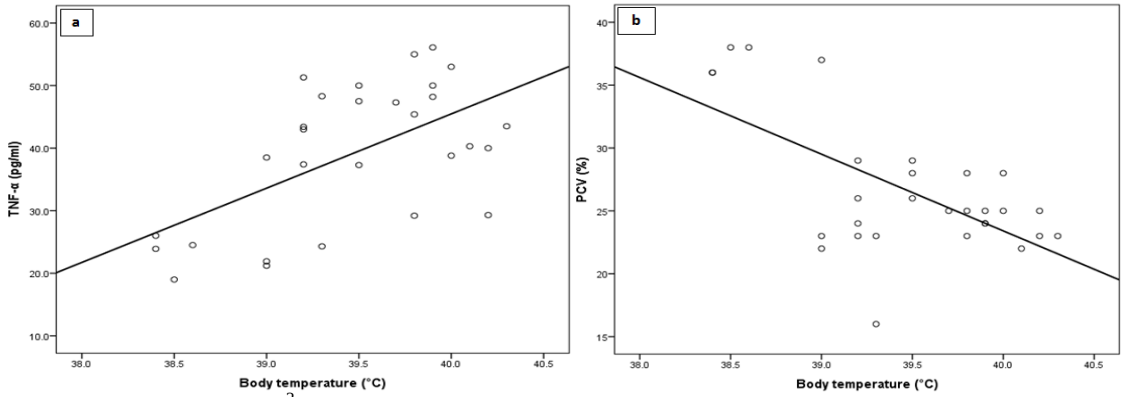
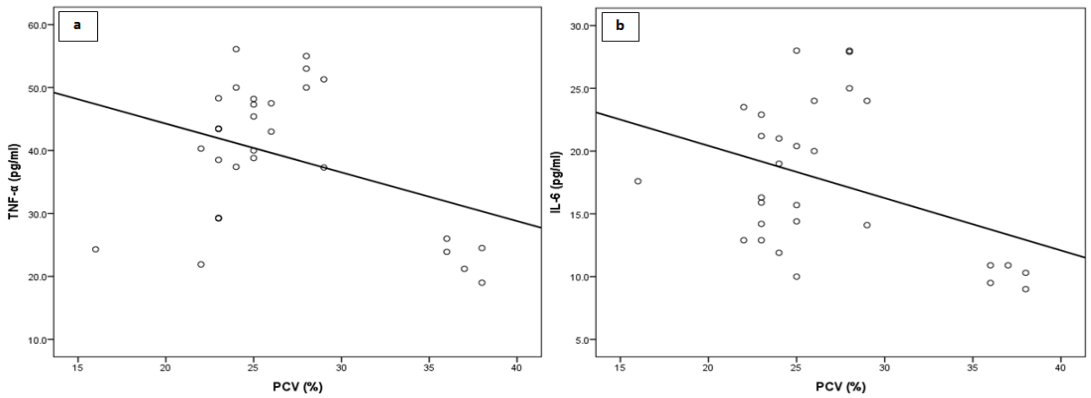


Fig. V: Linear regression (R^2) of Body temperature with TNF-α (a) and PCV (b) in *T. evansi* infected rabbits



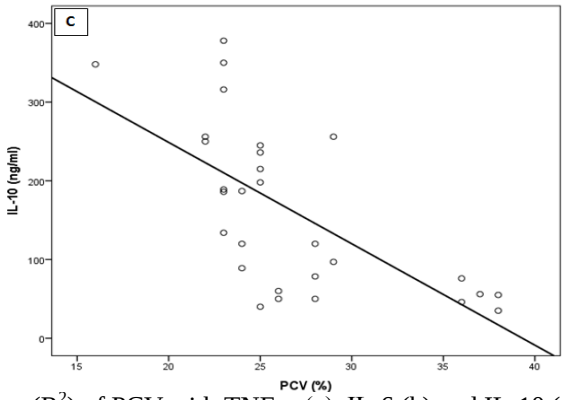


Fig. VI: The linear regression (R^2) of PCV with TNF-α (a), IL-6 (b) and IL-10 (c) in *T. evansi* infected rabbi

Table I: The clinical outcome along with the number and percentages of affected animals during the experimental period

Clinical features	Number of affected animals (n = 30)	Percentage (%)
Weakness	30	100
Anorexia	30	100
Lymphadenopathy	30	100
Pale mucosa	30	100
Orchitis	30	100
Alopecia of ear and above the nose	30	100
Corneal opacity	10	33.3
Blepharitis	10	33.3
Buffy eye	6	20
Facial oedema	5	16.7
Mortality	5	16.7

Table II: The body weight of rabbits during the experimental period (mean±S.D)

Days p.i.	Months p.i.	Mean rabbit weight (gram±SD) during days p.i.	
		Control	Infected
0	First	2130±109.54	2095±64.79
10		2140±74.16	2091±67.06
20		2140±65.19[a]	1963±117.4[b]
30		2150±50[a]	1960±100.34[b]
40	Second	2160±82.16[a]	1940±98.61[b]
50		2180±57.01[a]	1948±90.69[b]
60		2210±41.83[a]	1943.75±88.85[b]
70	Third	2230±57.01[a]	1941.67±88.06[b]
80		2280±44.74[a]	1945±62.62[b]
90		2310±65.19[a]	1917.5±81.56[b]
100	Fourth	2320±44.72[a]	1910±82.08[b]
110		2380±57.01[a]	1953.33±48.06[b]
120		2440±54.77[a]	1940±66.01[b]
130	Fifth	2510±41.83[a]	1928.57±87.08[b]
140		2550±93.54[a]	1937.5±130.25[b]
150		2580±90.83[a]	1937.5±127.48[b]
160	Sixth	2660±65.19[a]	1975±140.54[b]
170		2730±67.08[a]	2033.33±76.38[b]
180		2770±44.72[a]	2050±100[b]

[a b]within rows, values bearing different superscripts differ at P<0.0001

Table III: The time and haematological parameters of rabbits that died* or euthanized[&] during the experimental period

Group	Time of death (day p.i.)	RBC (×10^{12}/L)	Hb (g/L)	PCV (%)
G3[*]	86	1.8	42	15
G4[&]	110	1.8	40	14
G5[&]	140	1.79	48.2	16
G6[&]	165	1.84	43	14
G6[&]	165	1.97	46	15