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Immune Responses of Goats Infected with *Trypanosoma evansi* to Intranasal Pneumonic Mannheimiosis Vaccination

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Abstract:

Background*: A study was conducted to investigate the immunosuppressive effect of Trypanosoma evansi in goats given Intranasal Pneumonic Mannheimia (IPM) vaccination.*

Materials and Methods*: Twenty male goats were divided equally into four groups. Groups 1 and 2 were inoculated intravenously with 10^4 trypanosomes per animal, while goats in groups 3 and 4 served as uninfected vaccinated and uninfected unvaccinated controls, respectively. Goats in group 2 were treated with diminazine aceturate two days before primary vaccination. Groups 1, 2 and 3 received intranasal spray of 1 ml of Pneumonic Mannheimia (IPM) vaccine on day 30 post infection (PI) and a booster dose on day 44 PI. On day 58 PI all goats were inoculated intratracheally with 4 ml of live Mannheimia haemolytica organisms (10^6/ml) each. Blood samples were collected weekly and analyzed for IgG levels using antibody-ELISA assay. All goats were killed on day 72 PI, lung lavage fluid was collected and analyzed for IgA levels, while lung lesions were assessed grossly. All infected goats became positive with T. evansi between day 3 and 4 post infection. Group 1 remained positive throughout the experiment while Group 2 goats were negative 24 hours after treatment until the end of the experiment.*

Results*: All the goats survived the experiment except for one in group 3 which died before the challenge infection due to an unrelated cause. No significant difference ($P > 0.05$) was found in lung IgA levels nor lung lesion scores between the groups. Although serum IgG levels between the four groups did not differ significantly, highest IgG level was detected in the vaccinated group with the lowest level in the T. evansi infected group.*

Conclusion: *Thus the IgG findings imply that T. evansi in the early stage of the infection compromised the systemic immune response of intranasally vaccinated goats.*

Key Word*: Trypanosoma evansi; Mannheimia haemolytica; Immune response; Goat.*

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I. Introduction

Trypanosomosis due to *Trypansoma evansi* infection or surra and pneumonic mannheimiosis are important diseases of ruminants associated with high morbidities and mortalities in several parts of the world, causing serious economic losses (Zamri-Saad et al., 1996; Reid, 2002). The immunosuppressive effect of surra can lead to secondary infections and vaccination failure in susceptible animal species, which include horses, camels, dogs, cattle, buffaloes and goats (Luckins, 1988).

Recombinant intranasal pneumonic mannheimiosis vaccine possesses the advantages of direct stimulatory effect on the lung's mucosal immunity and the ease of administration seems to be more efficient in protecting animals against mannheimiosis than the earlier traditional parenterally administered pneumonia vaccines. Nevertheless, several factors such as stress, concurrent diseases and poor vaccination programmes were thought to aid in the immune compromise of vaccinated animals (Zamri-Saad et al., 1999; Zamri-Saad and Sabri, 2008). Thus, it was hypothesized that *T. evansi* infection could lead to immunosuppression and compromise the immune response of goats to intranasal pneumonic mannheimiosis vaccination. Therefore, this study was designed to investigate the effect of *T. evansi* infection on the cellular and antibody responses to pneumonic mannheimiosis following intranasal vaccination against *Mannheimia haemolytica* A2 infection.

II. Material And Methods

Study area and experimental animals

This study was undertaken at the Faculty of Veterinary Medicine, Universiti Putra Malaysia, Serdang, Selangor, Malaysia between April 2010 and July 2010.

Twenty healthy male local goats aged between six and ten months were procured from a goat farm. They have no history of trypanosomosis, manheimiosis and brucellosis. The goats were treated with Kelamectin 1% Plus® (KELA, Hoogstraten, Belgium) and Intracox® (Horsterweg, The Netherland), based on the manufacturer's recommendation to treat for gastrointestinal helminths, ectoparasites and coccidial infections, respectively. The goats were acclimatized to their new housing for four weeks before the commencement of the experiment. All goats were tested negative and confirmed free of *T.evansi* infection using the haematocrit centrifugation technique (HCT) (Woo, 1969) and CATT/*T. evansi* kit from Institute of Tropical Medicine, Antwerp, Belgium. The animals were kept in a fly-proof building with concrete floor throughout the study period, fed with commercial goat pellet and hay grass with water provided *ad libitum*. Rectal faeces and blood samples from the jugular vein were collected from each animal weekly to check for parasitic infections. A footbath containing 3% Lysol solution was provided at the entrance of the building.

Trypanosome isolates

The *T. evansi* strains used in the experiment were isolated in 2007 in Peninsular Malaysia from a deer (Deer isolate, Te033 from northern Perak state) during an investigation on surra-related mortalities on a deer farm (Adrian et al., 2010). The trypanosome was passaged four times in mice to increase the number of trypamastigotes per ml of blood and stored in liquid nitrogen until needed. The *T. evansi* (Te033) was lethal for mice within three to four days after inoculation. The trypanosomes were confirmed to be *T. evansi* by morphometry and molecular characterization using RoTat1.2 specific primers (Md-Isa, 2010). Before inoculation into the goats, *T. evansi* trypamastigotes were once again multiplied in mice. Infected mice were sacrificed when parasitaemia was high. *T. evansi* was counted in diluted mice blood using phosphate buffered saline in 10% glucose (PBSG) and counted in a Neubeuar haemocytometer (Dargantes et al., 2005). The volume of the diluent was adjusted to obtain a concentration of 10,000 trypomastigotes per ml.

Experimental design

Table 1 summarises the experimental design. The goats were allocated into four equal groups of five each based on their body weight.

Table 1. Experimental design for immunosuppressive effect of *T. evansi* in goats vaccinated with intranasal pneumonic mannheimiosis vaccine

Group	*T. evansi* (Day 0)	Therapy (day 28)	PPIMV (day 30)	BPIMV (day 44)	*M. haemolytica* (day 58)	Euthanasia (day 72)
1	Infected	None	PPMV	PPMV	Challenged	Done
2	Infected	Diminasan®	PPMV	PPMV	Challenged	Done
3	Uninfected	None	PPMV	PPMV	Challenged	Done
4	Uninfected	None	None	None	None	Done

Note: PPMV = Primary Intranasal Pneumonic Mannheimia vaccination; BPMV = Booster Intranasal Pneumonic Mannheimia vaccination; day = days post infection

At the start of the experiment, goats of Groups 1 and 2 received 1 ml suspension of the inoculum prepared earlier containing 10,000 *T. evansi* (Te033) trypomastigotes via jugular vein injection. The uninfected control Groups 3 and 4 received 1 ml of PBSG diluted blood of an uninfected mouse. On day 28 post-infection, goats of Group 2 were treated intramuscularly with 6.7mg/kg Diminasan 5%® (Diminazene diaceturate) based on the manufacturer's instruction. On day 30 PI, all goats of Groups 1, 2 and 3 were given 1 ml of primary intranasal spray vaccine against *Mannheimia haemolytica* A2 via the nostril. On day 44 PI, the vaccination was repeated on the same goats of Groups 1, 2 and 3, serving as a booster dose. Group 4 remained as uninfected and unvaccinated control. All goats were challenged intratracheally with $1.23X10^6$ cfu/ml of live *M. haemolytica* A2 on day 58 PI. Following challenge, clinical signs of pneumonic mannhemiosis were monitored on daily basis and scored according to Zamri-Saad et al. (1996) until the end of the experiment, when all surviving goats were killed.

At post-mortem, lung lesions were scored by a veterinary pathologist as described by Gilmour et al. (1983). Lung lavage fluid was collected immediately from all goats at post-mortem. Fifty ml of normal saline was introduced into the lungs via the trachea. After gentle massage of the lungs the fluid was drained and centrifuged for 15 min at 1000x*g* to remove any debris and stored at -20°C until used (Effendy, 1998).

Ethical approval

The procedures applied in this experiment were approved by the Animal Care and Use Committee, Faculty of Veterinary Medicine, Universiti Putra Malaysia.

Haematology and Serology

Four ml of blood sample was collected from each goat on day 0 PI and on weekly basis until the end of the experiment. Blood samples were taken for haematology using separate 21G X 1.5" needle in ethylenediaminetetraacetic acid (EDTA) anticoagulant coated Vacutainer® tubes.

Three ml of blood was collected from each goat on weekly basis from week 1 post-primary vaccination (PPV; day 44 PI) until the end of the experiment (week 6; day 72 PI). The blood was collected in plain Vacutainer® tubes using 21G X 1.5" needle. Serum was harvested from the blood after clotting by allowing the samples to stand for at least 3 hours at room temperature and centrifuged at 1000x*g* for 5 min to separate plasma from the blood. Serum samples were stored at -20°C until used.

Monitoring of infection

Trypanosoma evansi

Trypanosome counts were carried out immediately after blood sample collection throughout the experimental period. Enumeration of trypanosomes per ml was performed after diluting goat's blood with PBS using an improved Neubauer haemocytometer (Antoine-Moussiaux et al., 2009). When parasites were below the counting limit of the haemocytometer, trypanosomes were counted using the HCT technique of Woo (Wernery et al., 2001), by direct microscopic visualization at 100X magnification around the plasma buffy coat interphase of a blood sample after spinning at 1200x*g* for 5 min. The HCT technique was therefore used for simulation enumeration of trypanosomes and determination of percent packed cell volume (PCV) on a Hawksley haematocrit reader. Red blood cell count (RBC $X10^{12}$/L), Haemoglobin (Hgb g/L), total white blood cell count (WBC $X10^{9}$/L) were determined using an automated haematoanalyzer Cell-DYN® 1700; Division Abbott (Laboratories Abbott Park, USA). Percentage differential neutrophil, monocyte, lymphocyte, eosinophil and basophil were determined manually from Wright stained blood smears (Jain, 1993). Rectal temperature was recorded on weekly basis. In addition parasitaemia and temperature were determined on days 2, 4, 6 and 30 post-infection.

Serology

Serum IgG and lung IgA antibody levels against *M. haemolytica* A2 were determined using an in-house enzyme-linked immunosorbent assay (ELISA) technique (Zamri-Saad et al., 1996). Briefly, 50 µl of carbonate-bicarbonate buffer (pH 9.6) suspension containing $1X10^{6}$ killed whole cell *M.* (*Pasteurella*) *haemolytica* A2 was used to coat microtitre plates and incubated at 37°C over night. Diluted samples (1:40) were dispensed into the previously coated microtitre wells and incubated at 25°C for 3 hours in a shaker incubator. A diluted (1:8000) anti-goat IgG or IgA horseradish peroxidase conjugate was added to each well and incubated for two hours, after washing with PBS-Tween 20. Tetramethyl benzidine (TMB) substrate was added to each well for 30 min after washing. Finally the reaction was stopped by adding 2.5M sulphuric acid and the plates were read at 450nm. Each sample was run in triplicates. Data was excluded from the analysis when a value differed from the other two by 0.1 nm within the triplicate values.

Statistical analysis

Repeated continuous outcome variables (example rectal temperature) were analyzed using mixed model analysis of variance. Briefly, a repeated continuous variable was entered into a mixed model containing the main effect group (treatment), animal (nested in group), sampling days and the interaction group x days. Animal was considered as a random factor in the model. Probability (*P*) -value less than 0.05 was considered significantly different based on F-test (Lehman et al., 2005). One-way analysis of variance (ANOVA) or Kruskal-Wallis Tests was used to analyze continuous non-repeated data. All pairs using Steel-Dwass method comparison was used as non parametric post-hoc test. Parasitaemia values were log-transformed for normalization and graphical presentation of the data. The results are presented as least square means. Data analysis was performed in JMP, version 9.0.2 and 10.0.1, SAS Institute Inc., Cary, NC, (1989-2010).

III. Result

Clinical signs

T. evansi infection increased the rectal temperature, which was accompanied by parasitaemia in Groups 1 and 2. It started from day 4 PI and persisted until the goats were either treated or euthanized (Fig. 1). Highest mean rectal temperature (39.5°C) was recorded in Group 1. The appetite of infected goats was slightly reduced during the infection.

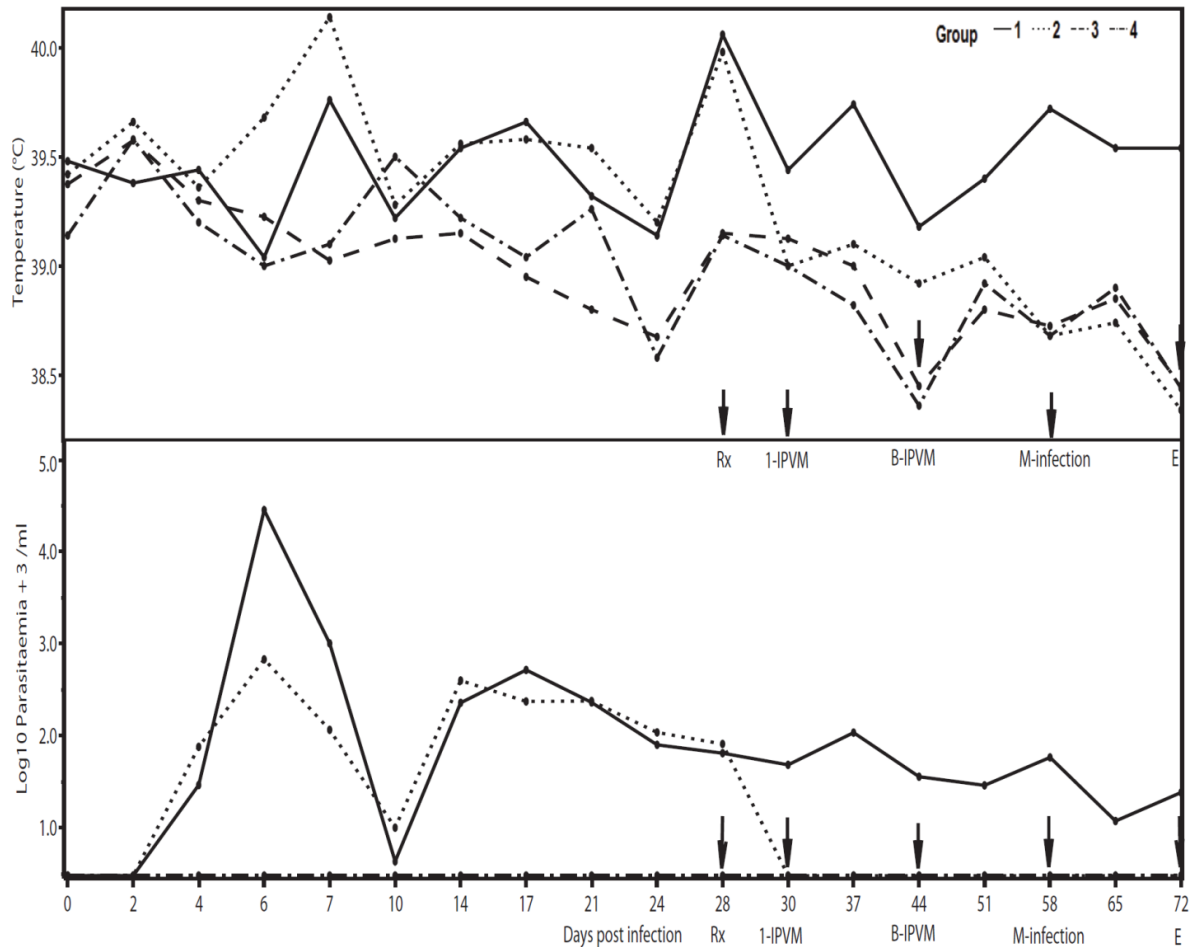

Figure 1: *Course and relationship of parasitaemia and rectal temperature of T. evansi infected (1), infected treated (2), uninfected vaccinated (3) and uninfected unvaccinated (4) groups of goats before, during and after M. haemolytica infection. Note: Rx = trypanocidal treatment of Group 2; 1-IPVM = Primary IPMV; B-IPVM = Booster vaccination; M-infection = M. haemolytica A2 infection; E = Euthanasia.*

On the other hand, following the bacterial challenge, the clinical signs among goats of Group 1 were marked more severe compared to other groups, but in general mild respiratory symptoms were noticed. The signs included intermittent coughing, watery nasal discharge and mucopurulent ocular discharge, which subsided after the first week of challenge. The temperature remained high until the end of the experiment but it was difficult to determine whether it was a result of single or the dual infection applied to Group 1. All goats survived the trial except one, which died from an unrelated cause. No significant difference ($P > 0.05$) was found in the lung lesion scores between the groups, which were generally mild.

Haematological changes

Red blood cell changes

Analysis of the red blood parameters showed that *T. evansi* infection caused significant ($P < 0.05$) decrease in PCV ($P < 0.01$), Hgb ($P < 0.05$) and RBC count ($P < 0.05$) in Groups 1 and 2. When the Group 2 goats were treated the parameters return to normal within two weeks post-infection (Fig. 2).

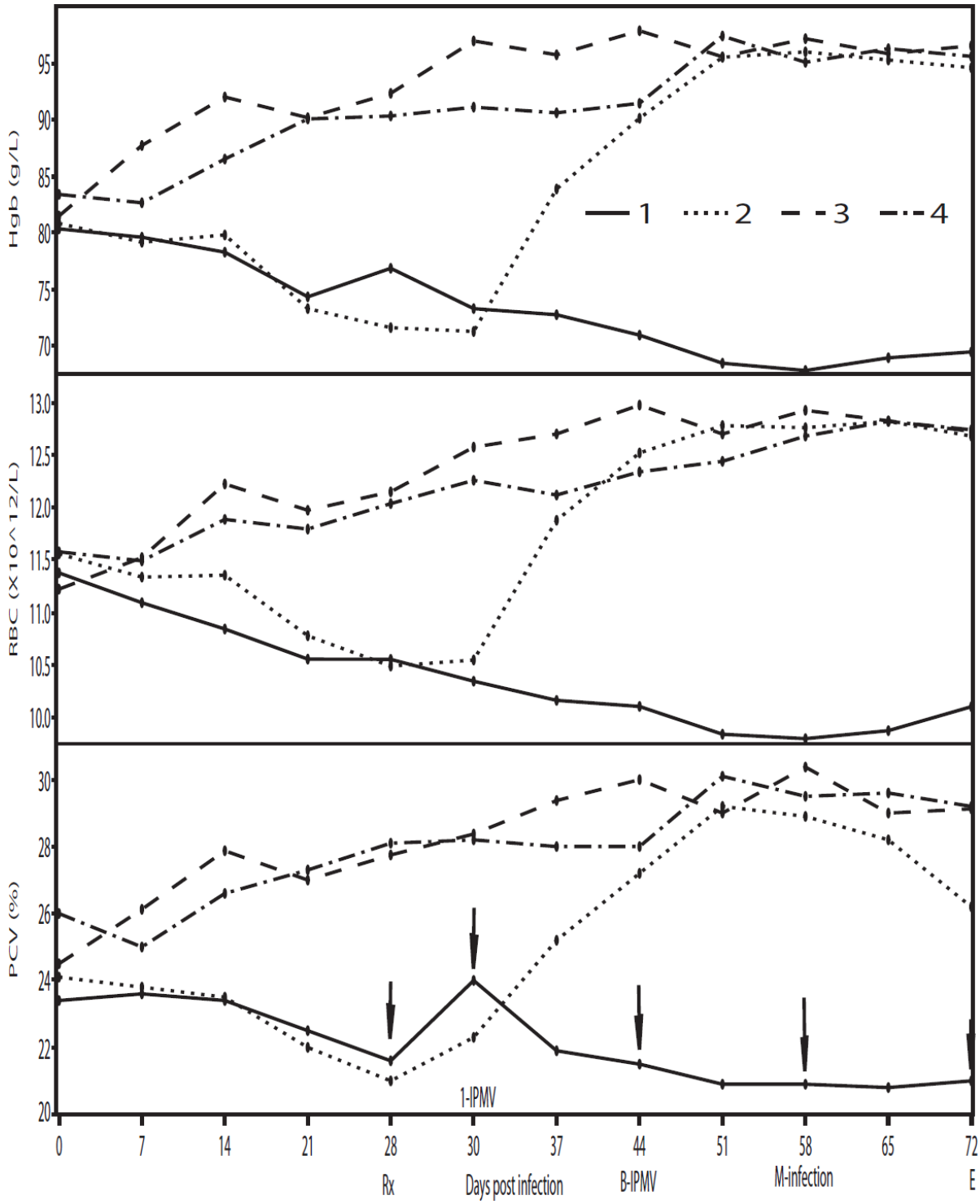

Figure 2: *Mean PCV (Packed cell volume), RBC (Red blood cell) and Hgb (Haemoglobin concentration) changes in T. evansi infected (1), infected treated (2), uninfected vaccinated (3) and uninfected unvaccinated (4) groups before, during and after M. haemolytica infection. Note: Rx = trypanocidal treatment of group 2; 1-IPVM = Primary IPMV; B- IPVM = Booster vaccination; M-infection = M. haemolytica A2 infection; E = Euthanasia.*

Total and differential white blood cell changes

The overall effect of *T. evansi* infection on the WBC parameters showed that monocytes ($P < .01$), neutrophils ($P < 0.05$) and basophil ($P < 0.05$) levels were significantly affected. Monocytosis, neutrophilia and basophilia were the main features observed (Fig. 2). Marked and sudden decrease in lymphocyte count followed by an increase in lymphocytes count was observed in Group 1 and Group 2 but not significantly different from control groups ($P < 0.05$) as shown in Fig. 3.

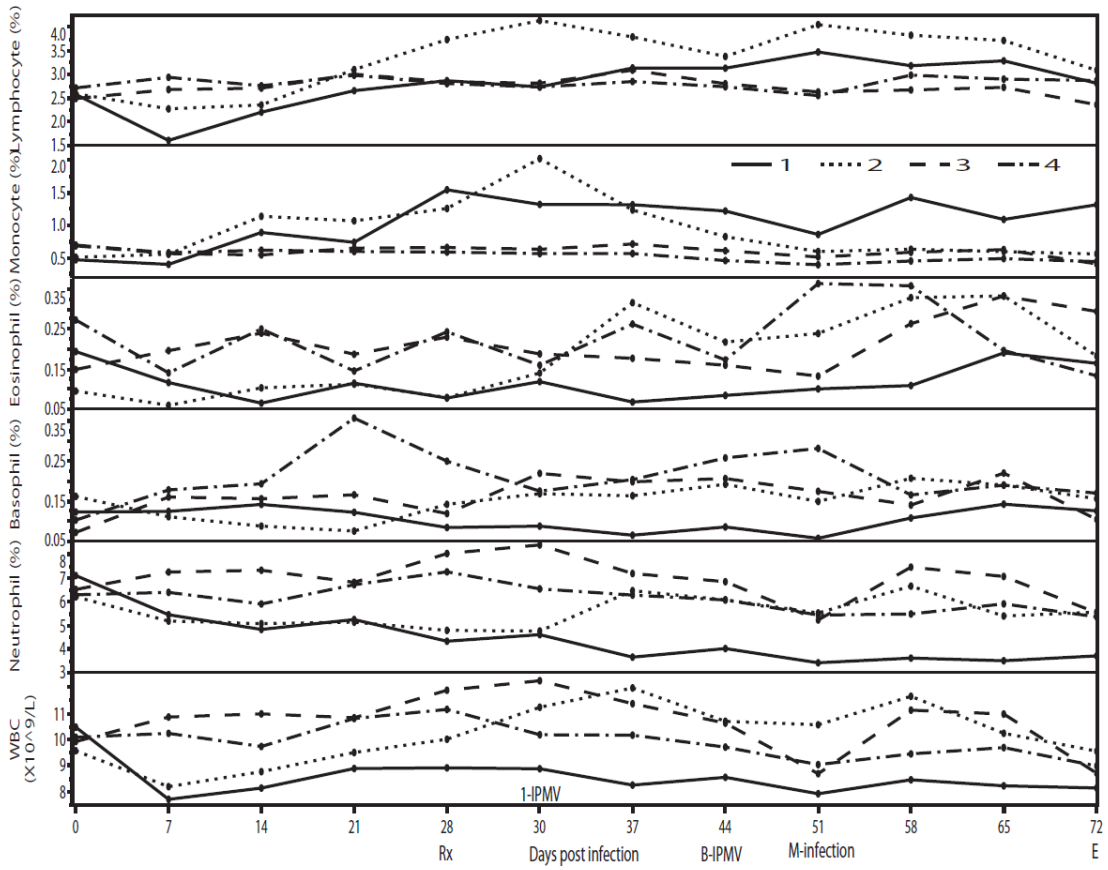

Figure 3: *White blood cell count and differential leukocyte counts of T. evansi infected (1), infected treated (2), uninfected vaccinated (3) and uninfected unvaccinated (4) groups before and after vaccination with IPMV.*

Lung IgA response

No significant difference ($P > 0.05$) was found between the mean lung IgA levels of the goats (Fig. 4).

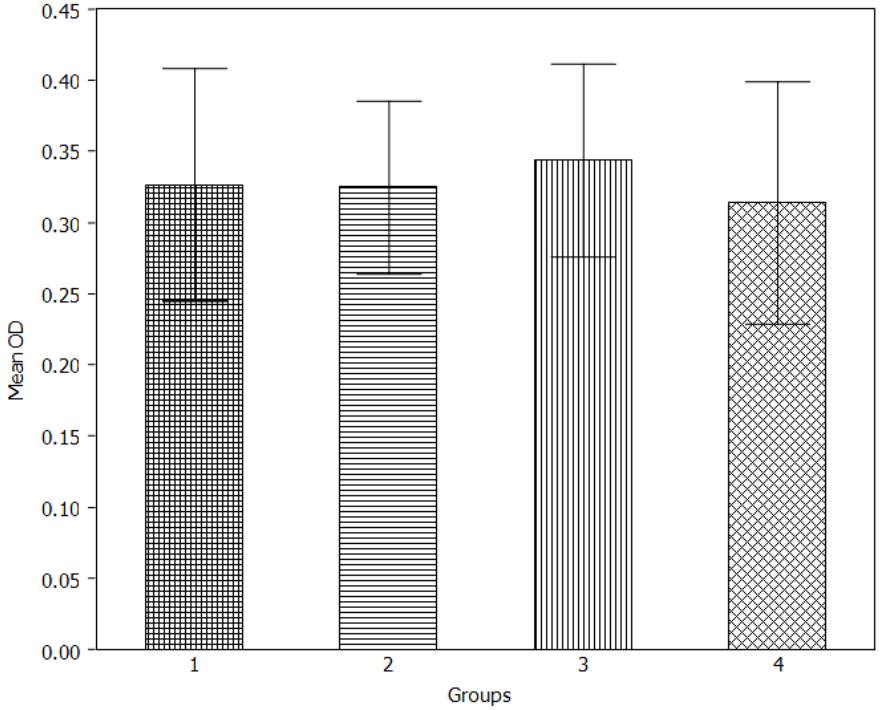

Figure 4: *Comparison of lung IgA responses between T. evansi infected (1), infected treated (2), uninfected vaccinated (3) and uninfected unvaccinated (4) groups. Each error bar was constructed using 1 standard error from the mean.*

Serum IgG response

Analysis of variance showed no significant difference ($P > 0.05$) in serum IgG levels between the groups (Fig.5). However, the trend in serum IgG responses indicated a continuous decline in only Group 1 from day 21 post-primary vaccination without any tendency of rising until the termination of the experiment.

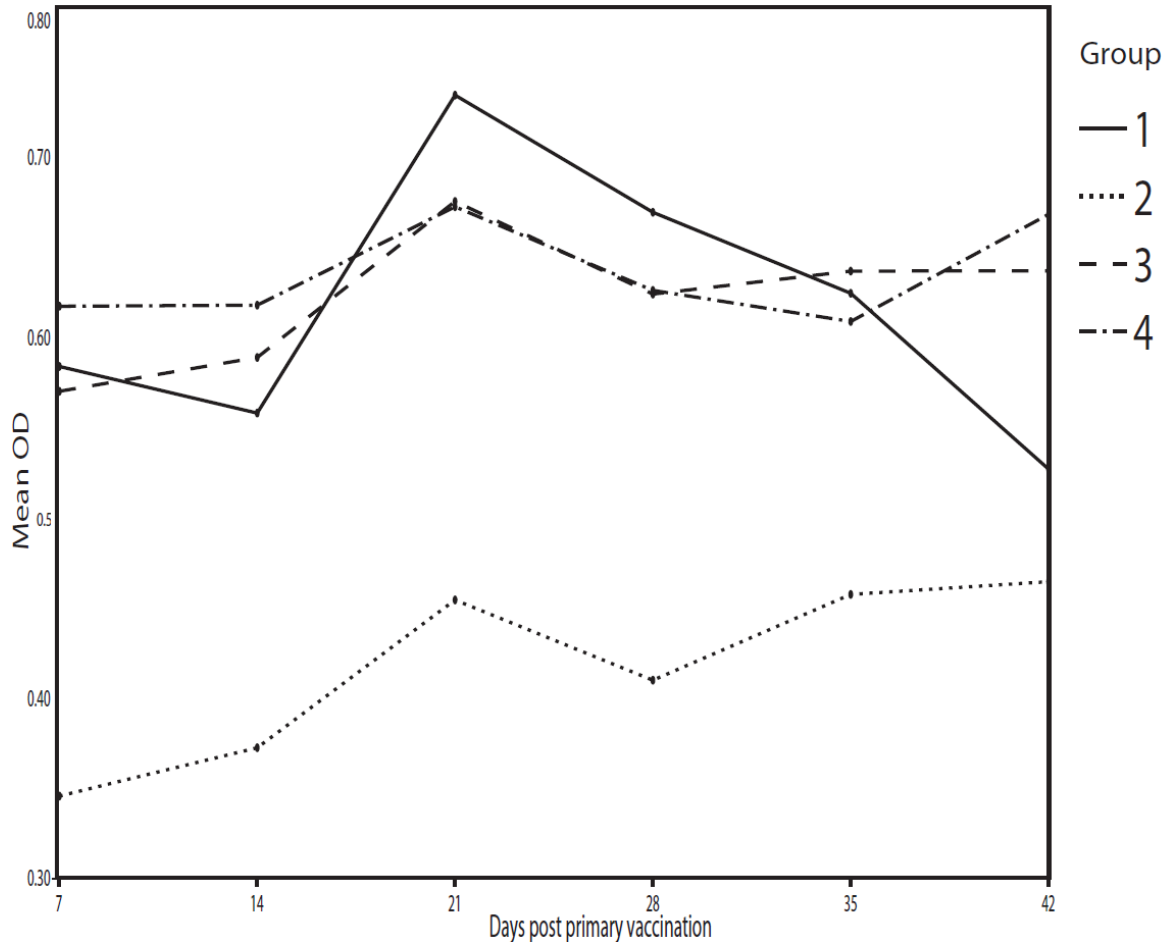

Figure 5: *Pattern of serum IgG responses between T. evansi- infected (1), infected treated (2), uninfected vaccinated (3) and uninfected unvaccinated (4) groups.*

IV. Discussion

The findings of this study showed that the deer isolate of *T. evansi* was capable of inducing clinical and haematological changes in the goats though not very severely. The pattern of parasitaemia was characterized by two major peaks and subsequent low levels, which persisted until the end of the experiment. Few studies have associated clinical and haematological parameters with immune response to vaccine in *T. evansi* infection studies. These studies include buffalo calves (Singla et al., 2010), cattle (Ikeme et al., 1984), goats (Shien and Wang, 1982) and mice (Antoine-Moussiaux et al., 2009). No significant effect of *T. evansi* infection on PCV was observed by Ikeme et al. (1984), which the authors related to the low level of parasitaemia experienced by the infected cattle after three weeks of infection. Shien and Wang (1982) and Singla et al. (2010) reported significant decrease in peripheral T lymphocyte response associated with chronic *T. evansi* infection in goats and buffalo calves, respectively. Antoine-Moussiaux et al. (2009) demonstrated in mice and *in vitro* that the mechanism of *T. evansi* lymphocytoxicity was caused by a membrane lymphotoxin (a 30 kDa protein sensitive to serine proteases) expressed by the parasite during exponential phase of growth. This finding supports the hypothesis of Rurangirwa et al. (1983) that the presence of active and persistent (but relatively high) parasitaemia is a prerequisite to the induction of immunosuppression by trypanosomes in their host. Therefore, the statistically insignificant difference in lymphocyte changes observed in our study could be associated with the early development of low parasitaemia following infection.

Suppression of the antibody response to vaccine is a common feature of trypanosomiosis (Losos, 1986) but not a rule. The results of our study showed that *T. evansi* did not cause statistically significant immunosuppression of lung IgA and serum IgG of goats to IPMV after 30 days PI despite persistent parasitaemia. This finding corroborates with the findings of Rurangirwa et al. (1983) and Taracha et al. (1987).

Chronic experimental *T. congolense* infection did not prevent cattle from mounting an effective antibody response to *Theileria parva* (East Cost Fever) infection after immunization and resisting subsequent lethal challenge compared to uninfected control (Taracha et al., 1987). Similarly, Rurangirwa et al. (1983) observed no depression of serum IgG and IgM responses to *Brucella abortus* vaccine (S19) in cattle chronically infected (25 weeks) with *T. vivax*. These studies and that of Ikeme et al. (1984) were all characterized by low and intermittent parasitaemia.

The lung IgA response of a group of *T. evansi* infected goats given trypanocidal treatment before vaccination in our experiment did not differ from the infected untreated goats. However, trypanocidal treatment seemed to enhance the serum IgG response. This is in agreement with the report of Ikeme et al. (1984) in an experimental infection of cattle with *T. evansi* subsequent to the haemorrhagic septicemia vaccination. The tendency of variation in the lung IgA and serum IgG responses observed in this study could imply that different mechanisms of immunosuppression may be operating at the peripheral blood and local lung mucosal immune system.

V. Conclusion

The present investigation using the deer isolate of *T. evansi* indicates that surra did not prevent goats from exerting adequate immunological response following IPMV immunization. This may not be the case under natural field conditions where surra may interact with other stress factors such as concurrent diseases, pregnancy, malnutrition and extreme weather conditions. These factors could aid in compromising the immunological status of the animals making them poor responders to vaccination. Therefore, further studies need to be undertaken using different doses and isolates of *T. evansi* in relation to other risk factors.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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