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Early stage leucocytosis in Nigerian pigs experimentally infected with *Trypanosoma brucei*

J. N. ABENGA [1*], S. ADAMU [2], N.M. USEH [2], A.J. NOK [3], N.D.G. IBRAHIM [2], A.K.B. SACKEY [4] and K.A.N. ESIEVO [2]

[1]*Department of Veterinary Pathology and Microbiology, College of Veterinary Medicine, University of Agriculture, Makurdi, Nigeria.*
[2]*Department of Veterinary Pathology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria.*
[3]*Department of Biochemistry, Faculty of Science, Ahmadu Bello University, Zaria, Nigeria.*
[4]*Department of Veterinary Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria.*
[*] *Corresponding author; E-mail: jnabenga@yahoo.com; Tel : +2348035877411 or +2347056574343*

ABSTRACT

Sequential leukocyte changes associated with early phase of *Trypanosoma brucei* infection were investigated in indigenous Nigerian pigs. This was with the view to providing further hematological basis for effective chemotherapy of natural porcine trypanosomosis and to assessing the possible roles of leukocytes in determining tolerance to infecting trypanosomes in this early phase of infection, earlier believed to be a crisis period for most infected animals. The parameters measured included, daily body temperatures and weekly body weights as indicators of onset of clinical aspect of the disease as well as total and differential white blood cell (WBC) counts. After infection, parasitemia was first detected in the pigs on day 3 post-infection (PI) which was accompanied by onset of hyperthermia, manifested as rise in rectal temperatures on the same day and afterwards by undulating increase throughout the 40 day observation period. The weekly body weights however showed fluctuating increase throughout this period. The total WBC counts of infected pigs increased progressively from the pre-infection value of 13.2±1.4 x 10^9 /L to the peak value of 38.5±12.6 x 10^9 /L (which corresponded to 191.7% increase above pre-infection values) on day 40 post infection (PI) while that of the control group only fluctuated within normal range. This was accompanied by an overwhelming, fluctuating surge in lymphocyte counts from the pre-infection value of 8.2±0.4 x 10^9 /L from day 1 to the peak value of 33.5±11.2 x 10^9 /L (corresponding to 308.5% increase above pre-infection values) on day 40 PI ($P<0.05$). The neutrophil counts also increased, though with a lesser intensity from the pre-infection value of 4.8±1.1 x 10^9 /L to the peak value of 6.6±3.3 x 10^9/L (corresponding to 37.5% increase above pre-infection values) on day 17 PI ($P<0.05$) which is at variance to the known hematological derangement patterns in most trypanosome infections. These were also accompanied by persistent eosinopenia but fluctuating monocytosis in the infected group. It was concluded that leukocyte derangement in the early phase of *T. brucei* infection of pigs was characterized by overwhelming lymphocytosis and was attributed to strain specific trypanosome antigenic challenge leading to an increased proliferation of immunocompetent cells into antibody and or lymphokine producing cells. This is believed to be beneficial to the host as it could serve as determinant of host tolerance to infecting trypanosome and ability to survive this crisis phase of *T. brucei* infections.

Keywords: Pigs, leukocytosis, lymphocytosis, *Trypanosoma brucei,* early phase.

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INTRODUCTION

Although pancytopenia constitutes a major hematological derangement feature of African trypanosomiases, nature and severity are often determined by specie, strain of infecting trypanosomes, parasitaemia and host factors (Mbaya et al., 2012; Kilekoung et al., 2014; Ukpai and Nwabuko, 2014) while the ability to control or resist the development of parasitemia and anemia had been identified as hallmarks of trypanotolerance in animals. *Trypanosoma brucei brucei* like other members of the *T. brucei* sub group, human infective, *T. brucei gambiense* and *T. b. rhodesiense* cause stage dependent pathology, characterized by early and late stage clinical diseases in animals that mimic sleeping sickness in man (Allam et al., 2011; Abenga, 2014) and are accompanied by variation in hematological changes depending on species and strain of trypanosome. Porcine trypanosomiasis is prevalent in sub-Saharan Africa where like in cattle (Abdoulmoumini et al., 2015; Mamoudou et al., 2016) results in deaths and 50% reduction in herd numbers (FAO, 2000). Although the true status of porcine trypanosomosis in Nigeria is not well known, results of spot surveys suggest that the disease is most prevalent in the Middle Belt and southern parts of Nigeria with the highest pig population density and trypanosome infection rates of at least 30% (Ogunsanmi et al., 2000; Omotainse et al., 2000). Economic losses arise from high mortalities, retarded growth, weight loss and abandonment of or failure to establish commercial pig farms in trypanosome and tsetse fly infested parts of the country (Swallow, 2000). Similarly, high cost of trypanocides and drug resistance; and relapse constitute sources of economic losses in the maintenance of pigs in high tsetse infested areas (Swallow 2000; Waiswa, 2005; Talaki et al., 2014).For many years, pigs have been identified as important reservoir hosts for African trypanosomes, especially *T.b. gambiense*, causative agent of sleeping sickness in West and Central Africa (Abenga and Lawal, 2005; Waiswa, 2005). Resurgence in the human disease in parts of Uganda, Equatorial Guinea (Simarro et al., 2001) and Cameroon (Nkinin et al., 2002) had been traced to pigs. Similarly, pigs had been associated with maintenance of old sleeping sickness endemic foci in Nigeria and has been identified as potential drivers of impending outbreak of the disease in other parts of the country (Abenga and Lawal, 2005). Molecular techniques suggest that human infections arise from maintenance of pigs – tsetse – human transmission cycle in endemic areas (Waiswa, et al., 2003). *T. brucei* has been described as a tissue invasive trypanosome (Talyor and Authie, 2004) with blood changes being secondary lesions. Characteristic lesions therefore arise from extensive inflammation of primary tissues affected with cellular infiltrations predominantly made up of lymphocytes followed by plasma cells, macrophages and neutrophils (Talyor and Authie, 2004). Microvascular injury and vasculitis (Abenga, 2014) are identified as important factors leading to increased vascular permeability, widespread embolism and thrombosis in small arteries and veins. Variation in research results of haemotological studies in *Trypanosoma brucei* in experimental infections pose a problem in the understanding of the pathogenesis and pathology of the disease in animals and man as *T. brucei* infected animals serve as models for sleeping sickness in man (Abenga and Anosa, 2006; Thuita et al., 2008; Ukpai and Nwabuko, 2014). Furthermore observations in pigs will help provide the needed consensus of opinion on the pathology of African trypanosomiasis and enhance its control. Early phase of infection is believed to constitute the crisis phase for most trypanosome infected animals as the host immune system may be

overcome and result to death of animals (Mbaya et al., 2012). Not much is known about the roles of leukocytes in trypanotolerance at this critical phase of infection. This study was therefore conceived to find the possible roles of leukocytes in determining the ability of infected pigs to tolerate the early phase of infection with *Trypanosoma brucei*.

MATERIALS AND METHODS

Experimental animals

A total of 15 growing pigs aged between 6 and 12 months old were used for the study. The animals were mixed breeds and aged as described by (Kahn, 2005). The animals were purchased from local pig farms within Zaria area and acclimatized for two months before the commencement of the experiment. During the acclimatization period, the pigs were dewormed using ivermectin (Kerpromec®, Deventer, Holland), a broad spectrum anthelminthic at the dose of 1 mℓ/50kg body weight and administered subcutaneously. The animals were also administered orally with, amprolium chloride, a broad-spectrum anticoccidial. Diazintol® (Diazinon dimpylate), a broad spectrum acaricide was fortnightly administered topically for ectoparasite control at the dose of 100 ml/2000 litres of water. The animals were housed in insect proof pens. The pens were thoroughly washed with water containing antiseptic and sprayed with acaricides. The animals were fed growing pig ration as described by FAO (2009).

Parasites used

Trypanosoma brucei, NITR/Federe strain obtained from the Nigerian Institute for Trypanosomiasis Research, Vom was used. The parasite history and handling had been described (Abenga et. al., 2015).

Experimental groups and infection of animals

The pigs were selected at random into two groups namely, the infected group made-up of 7 pigs and infected with 1 x 10^6 parasites (Lumsden,1972) in 2 ml of normal saline subcutaneously and the uninfected control group which was made up of 6 pigs.

Rectal temperature and body weight determination

The daily rectal temperature was obtained from each animal using clinical thermometer while weekly body weight was obtained using a Hanson balance, Emperors Marketing Company Limited, Lagos, Nigeria.

Parasitaemia

Wet blood films taken daily from the ear veins of infected pigs was examined under x 40 objective of a light microscope in order to confirm when the infection set in. 100 microscopic fields were examined before the result was declared negative when parasites were not detected.

Collection of blood for hematological procedures

Blood for hemogram and serum was obtained through venipuncture of the anterior vena cava using 19 gauge hypodermic needles and 10 ml syringes. The sites for the venipuncture were aseptically prepared by cleaning with 70% ethanol. Samples for regular hematology were collected into sample bottles containing Ethylene diaminetetraacetic acid (EDTA) as anticoagulant. Blood samples for serum separation were collected into clean test tubes containing no anticoagulant.

Evaluation of leukocyte parameters.

Total WBC counts were enumerated using Neubauer haemocytometer (Sood, 2006). For erythrocyte counts, the blood was diluted 1:200 with Dacies fluid (99 ml of 3%

aqueous solution of sodium citrate, and 1 ml of 40% formaldehyde) which keeps and preserves the shape of the red blood cells. For white blood cell counts, the blood was diluted 1:20 using 2% aqueous solution of acetic acid to which gentian violet was added. Thin blood smears stained with Giemsa (Sood, 2006) were used. 100 white blood cells were enumerated and differentiated per slide.

Statistical analysis

The data analysis was done using Graph Pad Prism Version 4.0 Software (Microsoft®). All data were expressed as mean ± SEM. Values between infected and control animals were compared using student t – test. Daily values within the infected group were compared using one way Analysis of Variance (ANOVA) and Tukeys Multiple Comparism Test. In all cases values of $P<0.05$ were considered significant. The procedures in this investigation were in line with the guidelines of the Ethical Committee of Ahmadu Bello University, Zaria, Nigeria.

RESULTS

Clinical changes in *T. brucei* -infected pigs

On day 0 of infection, the rectal temperatures of all experimental animals were identical ranging from 37.0 to 37.8 ^{0}C with mean values of 37.4 ± 0.2 ^{0}C and 37.5 ± 0.2 ^{0}C for the infected and control groups respectively. However, the rectal temperatures of the infected group increased to 39.5 ± 0.2 ^{0}C on day 3 which was also the first day of parasitemia and then was followed by undulating increases characterized by a peak values of 40.2 ± 0.7 ^{0}C ($P<0.03$) and 39.7 ± 0.7 ^{0}C on days 15 and 36 respectively (Figure 1) and then dropped to 38.4 ± 0.7 ^{0}C by day 40. The rectal temperatures of control groups varied within normal range. The body weight of all animals varied from 15.7 to 39.1 kg on day 0 before infection with the average of 22.2 ± 5.4 and 26.0 ± 7.7 kg for infected and control groups respectively. After infection the body weight of infected group increased progressively to 26.4 ± 5.0 kg by day 7 post-infection and thereafter dropped to values slightly above those of pre-infection over fourteen days and thereafter increased again to 27.4 ± 5.8 kg by day 40 even though it was not statistically significant ($P>0.05$). During this period, the mean body weight of control animals increased progressively to 36.7 ± 9.0 kg by day 40 (Figure 2).

Variation in leukocyte parameters

Total white blood cell counts

The mean total white blood cell (WBC) counts for infected and control groups on day 0 were 13.2 ± 1.4 and 13.4± 9.6 ($x10^9$/L) respectively while the range for all animals on the same day was 11.5 to 14.5 ($x10^9$/ul). After infection, the mean total WBC counts in the infected group showed an early but fluctuating increase after an initial drop on day 2 from day 3 with the first peak increase of 20.9 ± 7.3 ($x10^9$/L) occurring on day 7 in the first week of infection which was statistically significant ($P<0.05$). This was followed later by an apparent drop on day 9 but increased again thereafter with peak values of 29.9 ± 8.9, 35.6 ± 10.7, 33.9 ± 12.9 and 38.5 ± 12.6 ($x10^9$/L) in ascending order on days 17, 24, 33 and 40 respectively (Figure 3). These increases were also statistically significant ($P<0.05$). The total WBC counts of the control group only showed fluctuating but slight increase within normal range for growing pigs during the period.

Variation in differential leukocyte counts

Neutrophil Counts: Variations in the neutrophil counts of the animals are shown on

Figure 4. The mean neutrophil count of infected and control groups on day 0 was 4.8 ± 1.1 and 4.0 ± 0.8 ($x10^9$/L) respectively while the range for all animals on the same day was 3.3 to 6.4 ($x10^9$/L). After infection, the mean neutrophil counts in the infected group showed slight increase on day 1 followed by a drop on day 2 and then fluctuating increase, attaining the first peak value of 6.5 ± 1.8 ($x10^9$/L) on day 6 in the first week of infection which was statistically significant ($P<0.05$). This was followed by another drop in values below those of day 0 on day 9 when it progressively increased again to peak values 6.6 ± 3.3 and 6.4 ± 4.2 ($x10^9$/L) on days 17 and 21 respectively which were also statistically significant ($P<0.05$). Although the neutrophil counts thereafter showed fluctuating drop, they remained significantly high until day 40 when it dropped to values slightly above those of pre-infection. During this period, the neutrophil counts in the control group only showed slight fluctuating increase within the normal range.

Lymphocyte counts: Variation in the lymphocyte counts of infected and control pigs is shown on Figure 5. The lymphocyte counts in all animals varied from 5.8 to 9.2 ($x10^9$/L) on day 0 while the mean values for infected and control animals on the same day was 8.2 ± 0.4 and 7.6 ± 1.4 ($x10^9$/L) respectively. After infection, the mean lymphocyte counts of infected group also showed fluctuating increase from day 1 with the first peak value of 16.4 ± 7.1 ($x10^9$/L) occurring on day 7 and this was statistically significant ($P<0.05$). Thereafter, the lymphocyte counts showed an apparent drop on day 9 followed by progressive increase from day 13 to the second and third peak values of 34.6 ± 9.0 and 33.5 ± 11.2 ($x10^9$/L) on 24 and 40 respectively in ascending fashion. These increases were also statistically significant ($P<0.05$). The lymphocyte counts in control animals only showed slight but fluctuating increase during the period.

Eosinophil and monocyte counts: Variations in the eosinophil and monocyte counts of *T. brucei* infected and control pigs were as shown on Table I. The eosinophil counts of all animals ranged from 0.0 to 0.3 on day 0 while the average mean counts for infected and control groups were 0.0 ± 0.1 and 0.1 ± 0.1(x 10^9/L) respectively. After infection, the mean eosinophil counts in the infected group dropped significantly ($P<0.05$) throughout the infection period with only slight and sporadic increases to 0.0 ± 5.2 and 0.0 ± 9.9 ($x10^9$/L) on days 9 and 13 respectively.

The mean eosinophil counts of control animals showed fluctuating increases during the period. The variations in the monocyte counts of infected and control animals are also shown on Table I. Whereas the mean monocyte counts in infected and control groups were 0.0 ± 0.0 and 0.0 ± 0.0 ($x10^9$/L) on day 0 respectively, the variation in counts in all animals was 0.0 to 0.1 ($x10^9$/L) on the same day. After infection, the mean monocyte counts in the infected group showed fluctuating increase to the first peak value of 0.4 ± 0.6 ($x10^9$/L) on day 7 which was statistically significant ($P<0.05$). This was followed by an apparent drop on day 9 and a second peak increase of 0.29 ± 0.4 ($x10^9$/L) on day 13 which was also statistically significant ($P<0.05$). Thereafter, the monocyte counts only showed sporadic increase on day 33 post infection. During this period, the mean monocyte counts in the control group fluctuated within normal range.

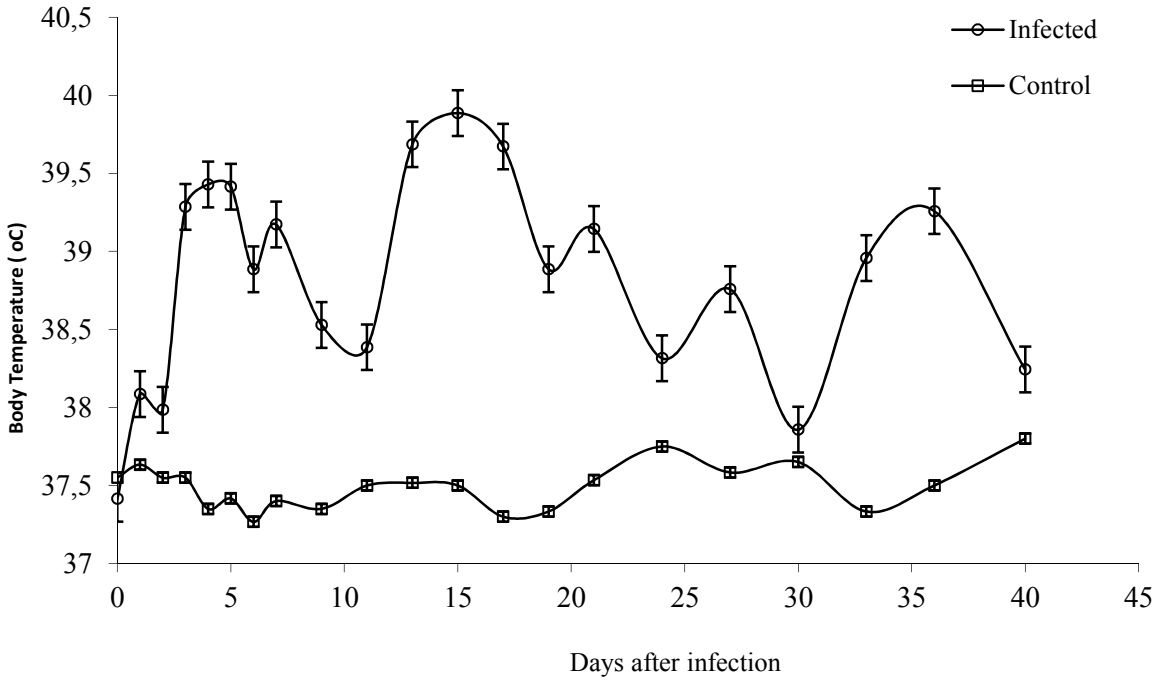

Figure 1: Variation in the mean body temperature of pigs infected with *T. brucei*

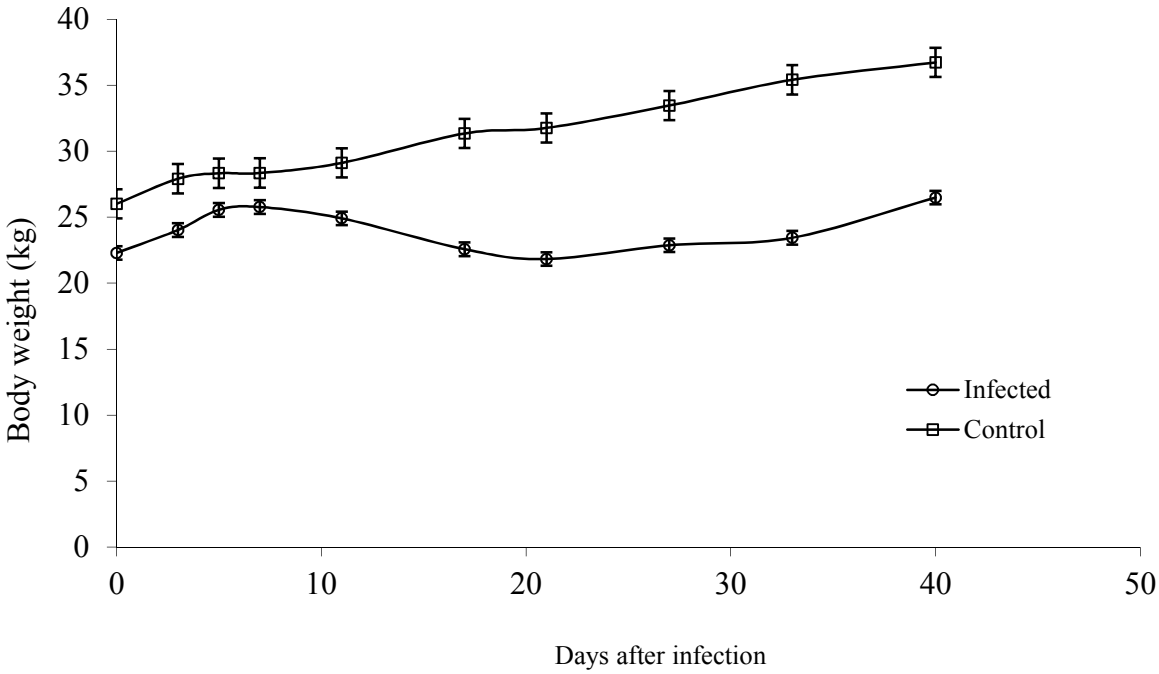

Figure 2: Variation in the mean body weight of pigs infected with *T. brucei*

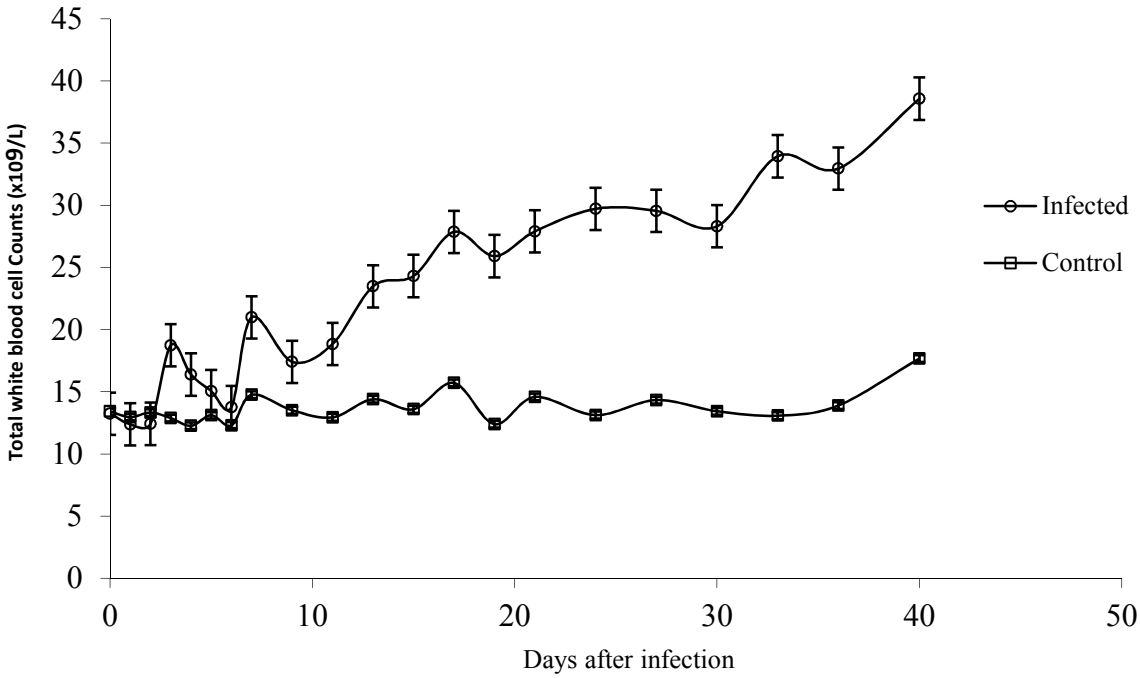

Figure 3: Variation in the mean total white blood cell counts of pigs infected with *T. brucei.*

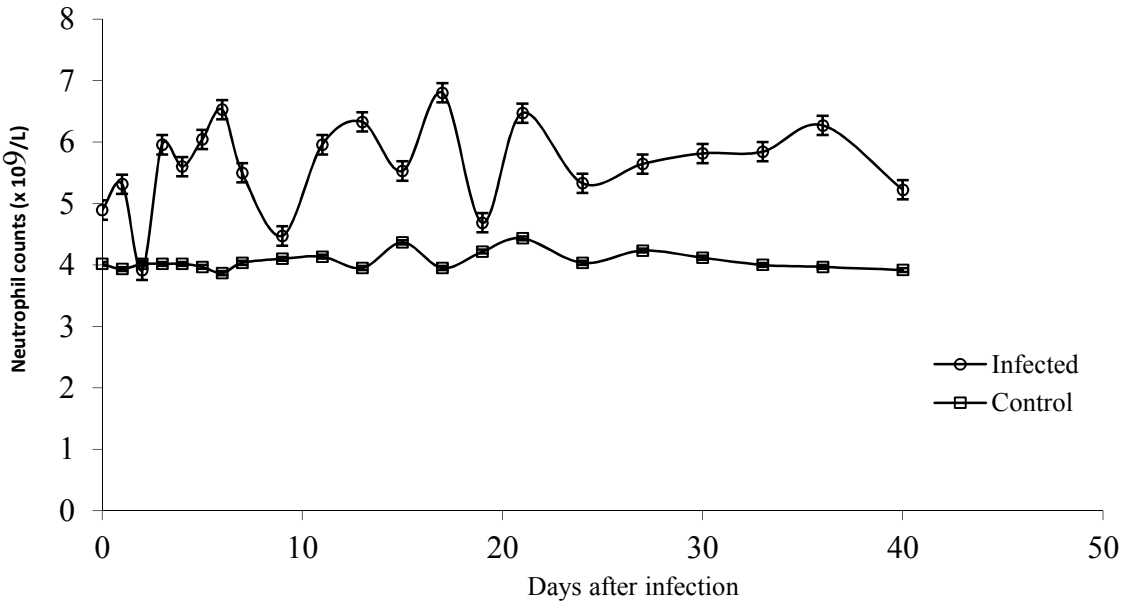

Figure 4: Variation in the mean neutrophil counts of pigs infected with *T.brucei.*

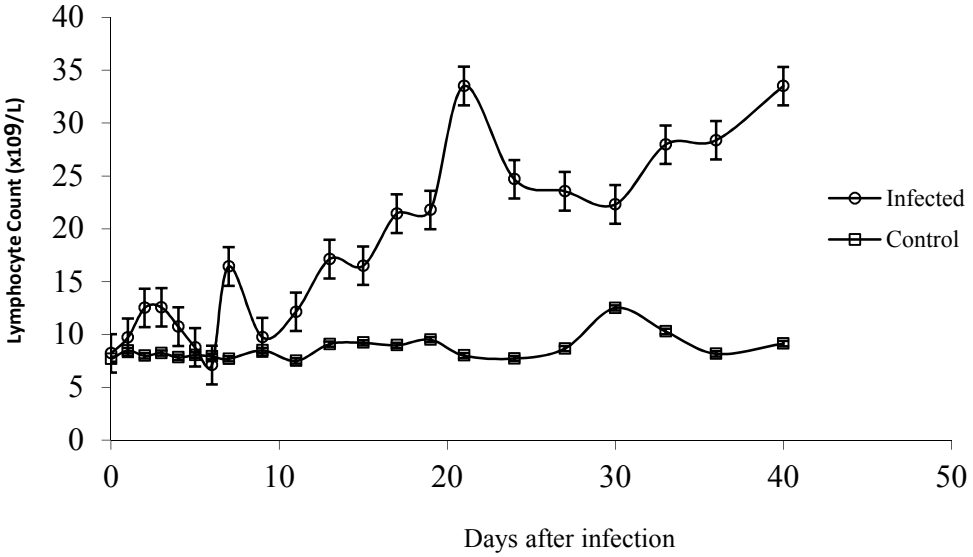

Figure 5: Variation in the Mean Lymphocyte counts of pigs infected with *T. brucei.*

Table 1: Table showing daily variation in the eosinophil and monocyte counts of *T. brucei* infected pigs and their control.

	Eosinophil counts (x10^9/L)		**Monocytes counts (x10^9/L)**	
Day	**Infected Group**	**Control Group**	**Infected Group**	**Control Group**
0	0.01± 0.00	0.16 ± 0.01	0.02± 0.01	0.02 ± 0.00
1	0.00 ± 0.00	0.01 ± 0.00	0.15 ± 0.01	0.00 ± 0.00
2	0.00 ± 0.00	0.20 ± 0.01	0.02 ± 0.01	0.00 ± 0.00
3	0.00 ± 0.00	0.10 ± 0.01	0.03 ± 0.02	0.00 ± 0.00
4	0.00 ± 0.00	0.24 ± 0.02	0.00 ± 0.00	0.00 ± 0.00
5	0.00 ± 0.00	0.16 ± 0.10	0.10 ± 0.01	0.01 ± 0.00
6	0.00 ± 0.00	0.00 ± 0.01	0.11 ± 0.02	0.00 ± 0.00
7	0.00 ± 0.00	0.05 ± 0.02	0.44 ± 0.10	0.00 ± 0.00
9	0.02 ± 0.00	0.51 ± 0.20	0.02 ± 0.01	0.00 ± 0.00
11	0.00 ± 0.00	0.21 ± 0.10	0.10 ± 0.10	0.00 ± 0.00
13	0.01 ± 0.00	0.10 ± 0.01	0.29 ± 0.05	0.01 ± 0.01
15	0.03 ± 0.01	0.01 ± 0.01	0.03 ± 0.02	0.00 ± 0.00
17	0.00 ± 0.00	0.00 ± 0.03	0.03 ± 0.03	0.02 ± 0.00
19	0.00 ± 0.00	0.12 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
21	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
24	0.00 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	0.00 ± 0.00
27	0.00 ± 0.00	0.15 ± 0.10	0.00 ± 0.00	0.00 ± 0.00
30	0.00 ± 0.00	0.20 ± 0.10	0.01 ± 0.01	0.01 ± 0.00
33	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
36	0.00 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.00 ± 0.00
40	0.00 ± 0.00	0.16 ± 0.10	0.00 ± 0.00	0.00 ± 0.00

DISCUSSION

The clinical feature of the *T. brucei* infection in the pigs was characterized by undulating increases in rectal temperature which also followed the parasitaemic pattern observed in the animals as the peaks in rectal temperatures also began to wane about the third week, similar to parasitaemic pattern in the infected pigs. These changes were consistent with previous observations in African trypanosomiasis (Taylor and Authie, 2004). Mbaya et al. (2012) earlier observed that high temperature contributes to red cell damage and development of anemia in Africa trypanosomiasis. Hyperthermia observed in this study may have accounted partly for the anemia and appearance of dullness observed in the *T. brucei* infected pigs. However, the body weight of infected animals did not drop significantly within the 40 days of observation, but rather began to increase in the last two weeks of the infection. *T. brucei* on the other hand caused early and remarkable increase in total WBC counts from day 7 post infection which was sustained throughout the infection period. These increases were due to marked and sustained lymphocytosis and neutrophilia. Although there was also an overall increase in monocyte counts, this may not have contributed significantly to this increase in the leucocyte counts. The eosinophil counts on the other hand decreased significantly in the infected group during the infection period. This pattern is at variance with pancytopenia characterized by leukocytopenia, neutropenia, lymphopenia and eosinopenia reported commonly in African trypanosomiasis (Allam et al., 2011; Taylor and Authie, 2004). These changes however agreed with recent observations of Yusuf et al. (2013) who also reported leukocytosis characterized by lymphocytosis, mild neutrophilia, eosinopenia and monocytosis in *T. brucei brucei* infected rats. Jibike and Anika (2003) also reported a similar and over whelming leukocyte response in *T. brucei* infected pigs which did not normalize with treatment with diffluoromethylorinithine alone and in combination with diminazine aceturate. Further support comes from Njiru et al. (2000) who reported similar observations in *T. evansi* infected dromedary camels. Hosts and trypanosome species may account for these variations in leukocyte responses (Taylor and Authie, 2004).

Whereas leukopenia occurs more frequently in African trypanosomiasis and had been associated with general depression of granulopoiesis, massive peripheral utilization, splenic sequestration and phagocytosis in other organs such as the bone marrow and the liver (Taylor and Authie, 2004), the etiology of leukocytosis in *T. brucei* infection had been tied to sustained lymphocytosis which is believed to result from trypanosome antigenic challenge leading to an increased proliferation of immune-competent cells into antibody and or lymphokine producing cells (Emeribe and Anosa, 1991). However this intense antigenic stimulation by the trypanosomes is capable of resulting in the depletion of the earlier hyperplastic lymphoid follicles and germinal centers resulting in ultimate lymphopenia in late stage of disease which had been associated with the loss of germinal centers in *T. brucei* infection of rats (Anosa, 1988). Whereas neutrophilia had been associated with an irritation of the bone marrow by *T. brucei* probably too weak to cause granulocyte hypoplasia (Emeriba and Anosa, 1991), this is likely also to arise from an early activation and proliferation of neutrophils by indirect mechanisms such as dispersal of

trypanosome antigens to the bone marrow and generation of tissue break down products due to extra vascular activities by trypanosomes. Monocytosis on the other hand had been reported to be matched by proliferation of macrophages in several tissues in trypanosome-infected animals whose proliferation and activation are believed to be stimulated by increased demands to remove particulate matter including trypanosomes, red blood cells, leukocytes and dead tissues cells (Anosa, 1988) *Trypanosoma brucei* being a tissues invasive parasite is therefore likely to generate more monocytes in circulation as the disease advances.

Conclusion

It is concluded that lymphocytic leukocytosis could enhance hosts' ability to tolerate and survive the early phase of *Trypanosoma brucei* infection in animals and man.

AUTHORS' CONTRIBUTIONS

JNA, SA, NMU and AJN designed the study and wrote the protocol; NDGI, AKBS and KANE supervised the work while JNA and NMU proofread the original draft of the manuscript.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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