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https://dx.doi.org/10.4314/nvj.v40i1.2 **ORIGINAL ARTICLE**

Trypanosoma congolense: Prophylactic Potentials Of Antiserum And Adjuvant In Experimental Mice

Chechet, G.D.[1*] and Aminu, R.U.[1]

[1] Department of Biochemistry, Ahmadu Bello University, Zaria.* Corresponding author: daglo2000@yahoo.com, mobile no:+23408094608793

ABSTRACT

Trypanosomiasis, a protozoan disease affecting livestock and transmitted by *Glossina* (tsetse) flies is a major constraint to livestock production in Sub-Saharan Africa. Approaches towards developing an anti-trypanosomal vaccine have so far shown little success. This study is targeted towards assessing the prophylactic potential of a "trypanotolerant" rabbit anti-serum vaccine in the presence or absence of an adjuvant (peanut oil) in the course of *Trypanosoma congolense* infection in mice. Forty healthy female Swiss albino mice were grouped in eight (*n=5*) as follows: unimmunized uninfected, serum-immunized uninfected, adjuvant-immunized uninfected, serum-adjuvant immunized uninfected, unimmunized infected, serum-immunized infected, adjuvant-immunized infected, serum-adjuvant immunized infected. For the first immunization, 200 µg of crude rabbit antiserum emulsified in a 100µl of phosphate-buffered saline (PBS) and adjuvant (1:1) or 200 µg of crude rabbit antiserum was injected intramuscularly on day 0. Similarly, the mice were administered a booster of half the crude antiserum (100 µg) every two weeks three consecutive times. Each mouse in the infected groups received 200µl of infective donor mouse blood containing $1x10^6$ Trypanosomes per ml of blood on day 42 post immunization. Blood samples were collected daily for parasitemia determination and on days 0, 42 and 62 post immunization for hematological parameters determination. The pre-patent periods, body weight changes and survival times were assessed as well. Immunization with the rabbit antiserum (emulsified with or without the adjuvant) resulted in significantly (P<0.05) lowered mean parasitemia levels. However, significant (P<0.05) increases were observed in the mean pre-patent periods, body weights, survival times, red blood cell counts and differential white blood cell counts in the serum and serum-adjuvant immunized groups. Results from this study have shown some promising effectiveness of antiserum prophylaxis when used alongside an adjuvant (peanut oil) during the course of *Trypanosoma congolense* infection in experimental mice.

Keywords:*Trypanosoma congolense*, Adjuvant, Anti-serum, Immunization, PCR

INTRODUCTION:

African trypanosomiasis is an infectious disease of humans and animals of similar aetiology and epidemiology. The causative agents of the disease are protozoan parasites of the genus *Trypanosoma* that live and multiply extracellularly in blood and tissue fluids of their mammalian hosts and are transmitted by the bite of infected tsetse flies (*Glossina spp*.). African animal trypanosomiasis or nagana disease is caused by *T. congolense*, *T. vivax* and *T. brucei* species. In wild animals, these parasites cause relatively mild infections while in domestic animals they cause a severe, often-fatal disease. All domestic animals can be affected by nagana and the symptoms are fever, listlessness, emaciation, hair loss, discharge from the eyes, oedema, anaemia, and paralysis. Other symptoms include lymphoid enlargement, loss of condition and immunosuppression with reduced host resistance to secondary infections (Darji *et al*., 1992).

Control of trypanosomiasis is based primarily on insecticide spraying to control tsetse populations and on regular treatment of livestock at risk with trypanocidal drugs. However, has complete control been achieved using these methods, even after substantial effort. The high cost of regular drug and insecticidal treatment, the limited effectiveness of insecticide application in high-rainfall areas, the possibility of environmental pollution by insecticides, the increasing incidence of parasite resistance to available drugs and the absence of new drugs to replace them are some of the problems that make tsetse and trypanosomiasis control difficult and expensive (WHO, 2005).

Other methods for controlling trypanosomiasis rely on the use of indigenous livestock such as N'Dama and other West African cattle breeds that are able to tolerate infection by trypanosomes. This genetic ability to resist pathogenic effects of infection is called trypanotolerance. The use of trypanotolerant breeds makes livestock rearing possible in areas where the presence of tsetse flies infected with trypanosomes preclude the production of animals that are susceptible to the disease (Murray *et al.,* 1990).

All attempts to produce vaccines against African trypanosomes were only partially successful or failed completely due to antigenic variation that has rendered the prospect of a plausible vaccine forlorn (Chechet *et al*., 2010; Magez *et al*., 2010).

Successful vaccination against a pathogen depends on the development in the host of a protective immune response that either neutralizes the effects of the pathogen or destroys it. When an animal is infected with trypanosomes, it produces immune responses to parts of the parasites. However, the immune responses in humans and most livestock types are unable to eliminate the parasites or even reduce parasite numbers to tolerable levels. The reason for this is that the trypanosome is able to change the molecules on its surface to which an animal's immune responses are normally directed (Field *et al.,* 2009).

Research results have shown that resistant animals such as the N'Dama cattle produce much better antibody responses to some components of the parasite than susceptible animals. The generation of antibodies to these parasite components appears to correlate with an ability to overcome infections. This correlation has significance for potential vaccine development (Silva *et al.,* 2009).

Adjuvants are essential immunostimulatory substances to stimulate the immunogenic response to antigens with low immunogenicity.

Freund´s adjuvant has been widely used over the years in experimental models, but the severity of its side effects have limited its use in vaccines for human beings and other animals (Gupta *et al*., 1993). Therefore, the development of new adjuvants is essential, not only on improving the low immunogenicity of purified antigens but also their promising influence on DNA vaccines (Sesardic and Dobbelaer, 2004)

In this study, we showed the immunogenic effect of emulsions prepared with trypanotolerant rabbit antiserum and peanut oil during the course of *Trypanosoma congolense* infection in experimentally infected mice.

MATERIALS AND METHODS

Parasites

T. congolense was isolated from an infected cow at an abattoir in Kaduna State, Nigeria and was characterized in the DFG laboratory at the Centre for Biotechnology Research and Training, Ahmadu Bello University, Zaria and passaged into albino rats. Cyclical transmission from tsetse flies to goats and serial passages in rats was used to maintain the parasites.

Parasite Authentication by Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) was performed using Phusion Blood direct kit (Finzymes) and ITS-1 generic primers to further authenticate the identity of the parasites. Briefly, 1µl of blood from an infected animal was included in the first round of PCR. The PCR was performed in a 20µl reaction volume containing 10µl of the Phusion buffer (Finzymes), 1.0µl of each outer primer (10 µM), 0.4µL of Phusion enzyme, 6.6µL of double distilled water and 1µL of blood that served as template in the first reaction. The cycling conditions were as follows: 1 cycle at 98^{0}C for 300 seconds followed by 35 cycles at 98^{0}C for 1 second, 57^{0}C for 5 seconds, 72^{0}C for 20 seconds and a final extension at 72^{0}C for 60 seconds. At the end of the reaction, the tubes were centrifuged at 1800 rpm for 3 minutes and 1µL of the supernatant was used as template in the second reaction. Nested PCR was performed using a 25µL reaction volume containing 1µL (1ng/µL) template DNA, 25µM of each primer, 10mM dNTPs, 25mM 10x Dream Taq polymerase buffer, 5U/µL Dream Taq polymerase to a reaction volume of 25µL. Cycling conditions were as follows: 1 cycle at 95^{0}C for 300 seconds followed by 30 cycles at 95^{0}C for 60 seconds, 56^{0}C for 60 seconds, 72^{0}C for 30 seconds, and final extension 72^{0}C for 220 seconds. The products were resolved by electrophoresis (45 minutes to 1 hour at 100V) on 1.0% Agarose gels stained with G-Stain (Serva®) and bands were visualized using GelDoc-It^2 Imaging System (Analytik Jena AG Jena, Germany).

Rabbit Serum

Serum used for immunization studies was obtained from a rabbit originally infected with a combination of *T.congolense* and *T.b.brucei*. After a period of time, *T.congolense* parasites were cleared from circulation and leaving *T.b.brucei*. The rabbit displayed waves of parasitemia and all clinical symptoms of Trypanosomiasis. Interestingly, the rabbit showed signs of recuperation after the 10 months period it survived the infection.

Experimental Animals

Forty healthy female Swiss albino mice of approximately 4 weeks old weighing between 12-26g were purchased from the National Institute of Trypanosomiasis Research, Kaduna. They were kept in cages lined with wood shavings and cleaned twice a week. The cages were also provided with water bottles to supply drinking water *ad libitum* to the mice. The mice were fed regularly with standard rat pallets. They were allowed to acclimatize for a

period of 2 weeks before the commencement of the experiment.

Determination of Parasitemia

Parasite count was determined microscopically at x40 magnification using the "Rapid Matching" method of Herbert and Lumsden 1976. Briefly, the method involves microscopic counting of parasites per field in pure blood or blood appropriately diluted with phosphate buffered saline (PBS, pH 7.4). Logarithmatric values of these counts obtained by matching with the table of Herbert and Lumsden (1976) is converted to antilog to provide absolute number of trypanosomes per ml of blood (Atawodi, 2003).

Immunization Studies

The mice were grouped randomly into 8 groups of 5 mice each as follows: Group 1-unimmunized uninfected, Group 2- serum-immunized uninfected, Group 3- adjuvant-immunized uninfected, Group 4- serum-adjuvant immunized uninfected, Group 5-unimmunized infected, Group 6- serum-immunized infected, Group 7- adjuvant-immunized infected, Group 8- serum-adjuvant immunized infected. All the groups of immunized mice received intramuscular injection in each thigh muscle of either rabbit serum (200µg/100µl), adjuvant (*Arachis* oil) 100µl, both in an equal ratio (50µl serum: 50µl adjuvant) or PBS on day 0 post immunization. Half the amount of serum (100 µg/100µl) was used to immunize the mice on day 14 and 28 post immunization. On day 42, all animals in the infected groups were infected by intra-peritoneal injection with $1x10^6$ savannah strain of *T. congolense*. Blood was collected in EDTA tubes at three different time intervals (Day 0, 42 and 62 representing pre-immunization, post-immunization and post-infection times respectively).

Determination of Hematological Parameters

Blood was collected at three different time intervals via each mouse-tail into EDTA tubes. Hematological analysis was performed using standard protocols. Differential leucocyte counts and Red Blood Cell (RBC) counts were determined manually employing an improved Haemocytometer. For the determination of differential leucocyte counts, Giemsa-stained thin blood smears were prepared and the number of cells per slide (usually 100 cells), the shape of the nucleus as well as the presence or absence of granules in their cytoplasm. The relative numbers of monocytes, basophils, neutrophils, eosinophils, lymphocytes and leukocytes were expressed as percentages and calculated as:

%=Number of cells inside differential cell area/Number of cells in total cell area x 100

Whereas, RBC counts in turn were expressed as number of cells per microliter of blood.

Determination of Body Weight

The weight of each animal was carefully determined using a digital weighing balance. The weights of the animals were taken at three intervals during the course of the experiment; pre-immunization which served as the baseline the baseline, post immunization and post infection.

Determination of Survival

Animal survival was determined daily post-infection and the mean survival time was calculated using the Kaplan-Meier survival curve.

Statistical Analysis

All data were expressed as means ± standard deviation of five measurements using Graph Pad Prism 7 software Version 7.0a. One-way ANOVA was used to assess the differences in pre-patent period. Two-way ANOVA was used to assess body weight as well as the hematological parameters. The level of

significance was set at ($p < 0.05$).

RESULTS

Pre-patent Period, Parasitemia and Survival Assessment

Species-specific primers were used to amplify the ITS-1 region of the gDNA and a strong band was produced at 850bp while the negative control didn't show any bands (Figure 1).

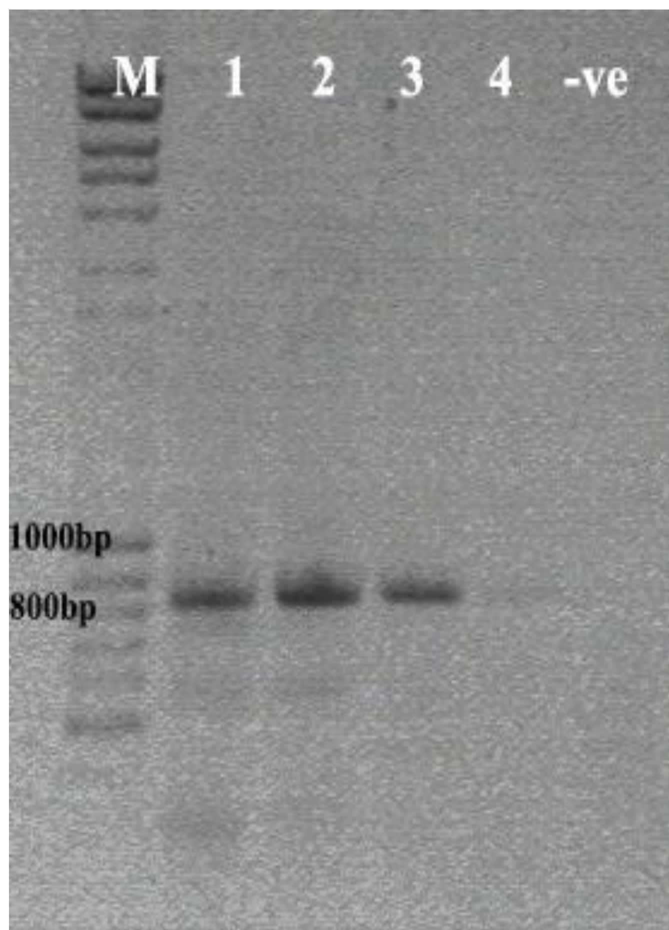

Figure 1: PCR identification of *T. congolense* using species-specific primers. The PCR products were analyzed by the electrophoresis on a 1.0% Agarose gel stained with G-Stain. M-Marker, Lane 1,2,3 - gDNA from *T.congolense* infected mice,-ve – nNegative control with water

Findings on the unimmunized infected group had a mean pre-patent period of 10.6±1.14 days. While animals immunized with serum-adjuvant, adjuvant alone and serum alone had mean pre-patent periods of 16.8±1.48, 9.4±1.14, 11±0.71 days post infection respectively as shown in figure 1. The pre-patent periods of the immunized adjuvant alone and serum alone were not significantly different ($P>0.05$) from that of the unimmunized infected group. The unimmunized infected and adjuvant groups developed parasitemia of 0.05±1.12 parasites/ml on days 9 and 8 respectively while the serum and serum-adjuvant immunized groups developed parasitemia of 0.10 ±1.37 parasites/ml each on days 11 and 17 respectively (Figure 2). The mean parasitemia peaked at 6.95±3.34 parasites/ml on day 20 for the unimmunized infected group while the mean parasitemia peaked at 5.02±1.79 parasites/ml on day 23 for the adjuvant-immunized group. However, for the adjuvant-immunized group, the mean parasitemia peaked 0.31±0.11 parasites/ml on day 22, declined to 0.05±1.12 parasites/ml on day 23 but increased to 0.20±1.12 parasites/ml on day 24 and peaked at 1.38±1.89 parasites/ml on day 31. On the other hand, the serum-adjuvant immunized group peaked at 0.28±0.11 parasites/ml on day 24, declined the level where no parasites were observed on day 28 but parasitemia resurfaced on day 29 which increased and peaked on day 40 at 0.97±1.03 parasites/ml (Figure 2).

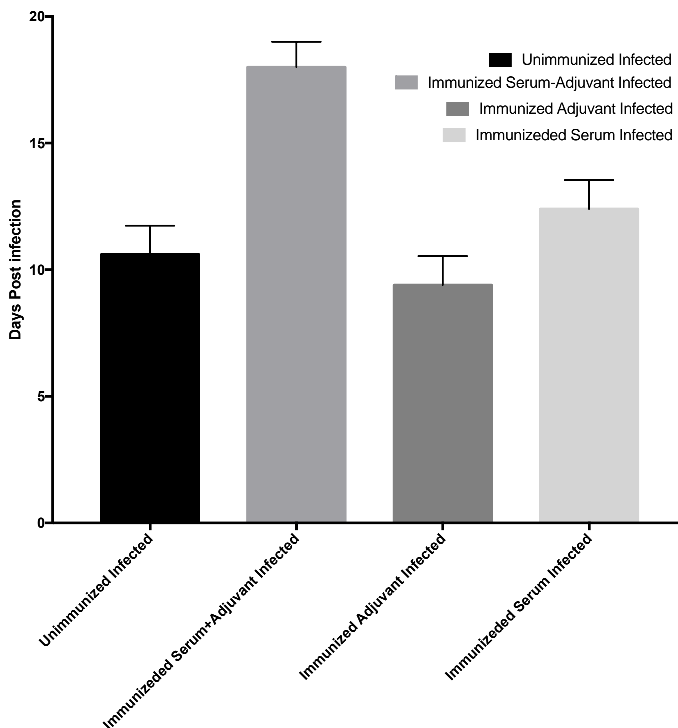

Figure 2: Mean pre-patent periods (days) of immunized and unimmunized infected groups of experimental mice.

Animal Survival and Body Weight

Mice in the unimmunized and adjuvant-immunized groups survived until days 13 and 16 respectively, after which they started to die. 60% of the animals from the same group survived until days 15 and 18 respectively, after which they all died at 21 and 24 days respectively. In contrast, animals in the serum and serum-adjuvant immunized groups survived until days 22 and 33 respectively before they started dying and 50% of them survived until day 28 and 38 respectively. 20% of the mice in both groups survived after 30 and 40 days respectively (Figure 3). Mean weight loss was shown to be more pronounced in the infected groups and highest in animals of the unimmunized untreated 18.2±1.48 and was statistically insignificant (P>0.05) when compared to mean weight of animals (at the termination of the experiment) in the serum-immunized 18.8±2.49 and serum-adjuvant immunized groups 20.4±2.30 (Figure 4). Also, a decrease in mean weight of 21.4±2.30 to 18.8±2.49 was also observed in animals of the serum-immunized as well as in serum-adjuvant immunized group from 24.00±1.00 to 20.4±2.30. In contrast, a steady increase in body weight was observed in the uninfected groups throughout the duration of the experiment.

Hematological Parameters

Findings showed there was no significant difference (P>0.05) in mean RBC count between the infected and uninfected groups before and after immunization (Figure 6). However, after infection, there was no significant difference (P>0.05) in mean RBC count between the unimmunized infected group with adjuvant-immunized groups. However, there was a significant difference (P<0.05) in mean RBC counts between the unimmunized infected, serum and serum-adjuvant immunized groups. Similarly, there was a significant reduction in RBC count between the serum and serum-adjuvant immunized groups but none was observed between the serum immunized and adjuvant immunized or serum-adjuvant immunized groups.

The mean differential WBC count indicated that the basophils, eosinophils, monocytes neutrophils and lymphocytes fluctuated within control levels in all groups during the pre and post immunization periods of the experiment (Figures 7-11). Significant (P<0.05) decreases in the mean differential WBC counts were observed in the animals of the infected immunized groups post infection and did not return to basal level when compared with the unimmunized infected group of animals. On the contrary, significant (P<0.05) monocytosis was observed in the infected group being most prominent in the unimmunized and adjuvant-immunized infected groups post infection.

Notably, animals in the unimmunized and adjuvant-immunized infected groups consistently showed the most depressed mean differential WBC counts (besides monocytes count) post infection when compared to animals in the other infected groups while the serum-adjuvant immunized group showed the highest elevation.

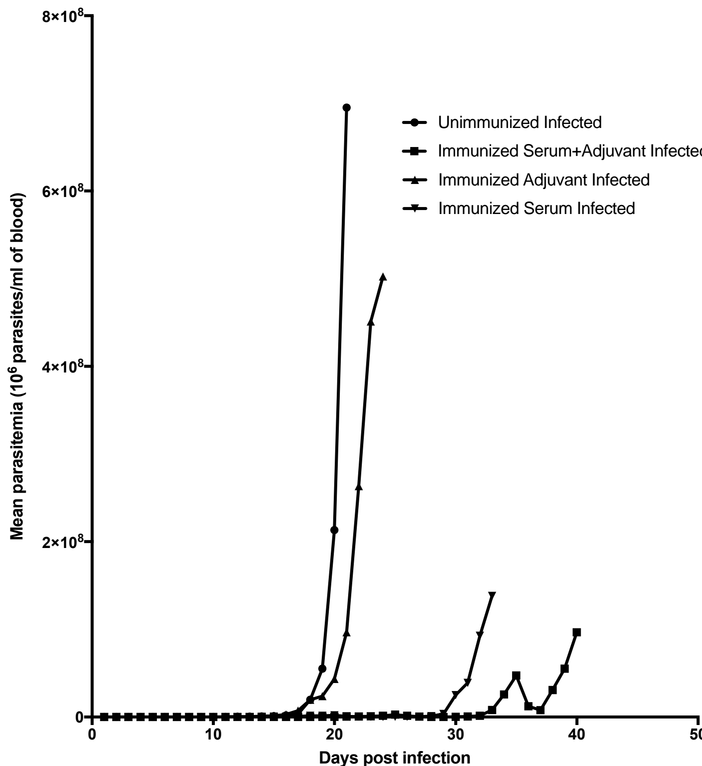

Figure 3: Mean parasitemia of immunized and unimmunized infected groups of experimental mice.

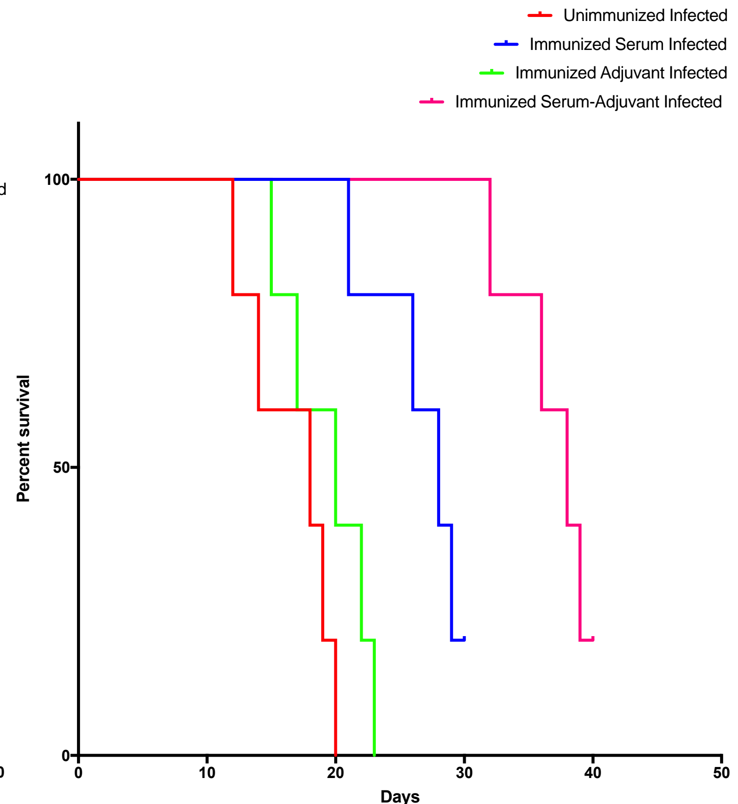

Figure 4: Kaplan-Meier mean survival plots for the immunized and unimmunized infected groups of experimental mice.

DISCUSSION

Mixed infections of trypanosomes occur in the field. Therefore, the precise identification of species used in experiments is essential for understanding the epidemiology and pathogenesis of these parasites (Gibson, 2007). Thus, it was imperative to detect the right *Trypanosoma congolense* specie used in this study. Microscopy has been the gold standard tool available for detection and identification of parasites in blood and other biological samples (Tanowitz *et al*., 1992; Herwaldt, 1999; Melrose *et al*., 2004; Duffy and Fried, 2005; Costa-juniour *et al*., 2012). Although this inexpensive diagnostic test has been proven sometimes, to be insensitive, inaccurate, despite its being inexpensive (Böse *et al*., 1995; Costa-juniour *et al*., 2012). We therefore employed a PCR-based approach that is sensitive and reliable for detecting and identifying various trypanosome species in blood through amplification of the ITS-1 region using two primer sets, which gave a unique

product size of 850bp for *T.congolense* savannah (Adams *et.al*., 2006).

From the results of this study we have demonstrated that immunizing Swiss albino mice challenged with of *T. congolense* ($1x10^6$) with serum of a trypanotolerant rabbit conferred some form of protection on the immunized-infected groups of mice. This was observed in the extended pre-patent period and decreased parasitemia levels observed in the groups immunized with serum and serum-adjuvant when compared to the groups that were immunized with the adjuvant only or non-immunized groups. The pre-patent period as well as parasitemia was observed to be extended in the serum-adjuvant immunized group compared to the serum immunized group.

A decreased RBC count was observed in infected mice even though the decline was most prominent in the unimmunized as well as the adjuvant-immunized groups. Anemia, which is the hallmark of trypanosomiasis infection (Noyes *et al*., 2009), is usually characterized by a decreased red blood cell count. Studies have suggested that galectin-3 (Gal-3) and macrophage migration inhibitory factor (MIF) possibly could be operating in concert with TNF-α during the development of anemia. While Gal-3 elevates erythrophagocytosis, MIF bolsters both myeloid cell recruitment and iron retention within the Mononuclear Phagocyte System (MPS), thereby divesting iron for erythropoiesis. Thus, the concomitant enhancement of erythrophagocytosis and suppression of erythropoiesis eventually leads to anemia (Stijlemans *et al*., 2018).

Besides anemia, another remarkable trait of African animal trypanosomiasis is immune system suppression of the infected mammalian host. Generation of macrophages that suppress both antibody mediated immunity (B cells) and cellular immunity (T cells) as well as polyclonal activation of B cells have both been hypothesized to account for immunosuppression during trypanosome infection in mice (Ademola and Odeniran, 2016).

Decreased numbers of basophils, eosinophils, neutrophils as well as lymphocytes were observed in the unimmunized infected groups post-infection when compared with the uninfected groups. In contrast, an elevated monocyte level was recorded in the infected groups. Our results corroborate the findings of Ademola and Odeniran (2016) and Dagnachew *et al*., (2015) who reported Leucopaenia, thrombocytopenia, lymphopaenia, neutropaenia, pancytopenia and monocytosis associated with *T.b.brucei*, *T.congolense* and *T.vivax* infections in mice and cattle. On the contrary, a consistent elevated level of the differential WBC counts (besides the monocytes) was observed in the immunized infected groups. These results suggest that the serum could actually have conferred some protection on the immunized infected animals and more so on animals that received the combination of serum and adjuvant which is seen by the boosts in differential WBC counts observed in the immunized infected groups. Neutrophils are among the first immune cells to travel to the infection site and are important in controlling the host defense through oxidant and protease-dependent mechanisms (Faurschou and Borregaard, 2003; Laskay *et al*., 2003; Nauseef, 2007). These cells also provide an important link between innate and adaptive immunity during parasite infections (Appelberg, 2007; Nathan, 2006). Similarly, Eosinophils are components of the immune system that fight multicellular parasites and are most often regarded as indicators of parasitic infections. Gleich and Adolph (1986) demonstrated, the participation of eosinophils in host defense mechanisms to helminth

infection and the in-vitro killing of various parasites by eosinophils from different species while Nakhle *et al*., (1989) demonstrated the participation of eosinophils *in vivo* during *T.cruzi* infection.

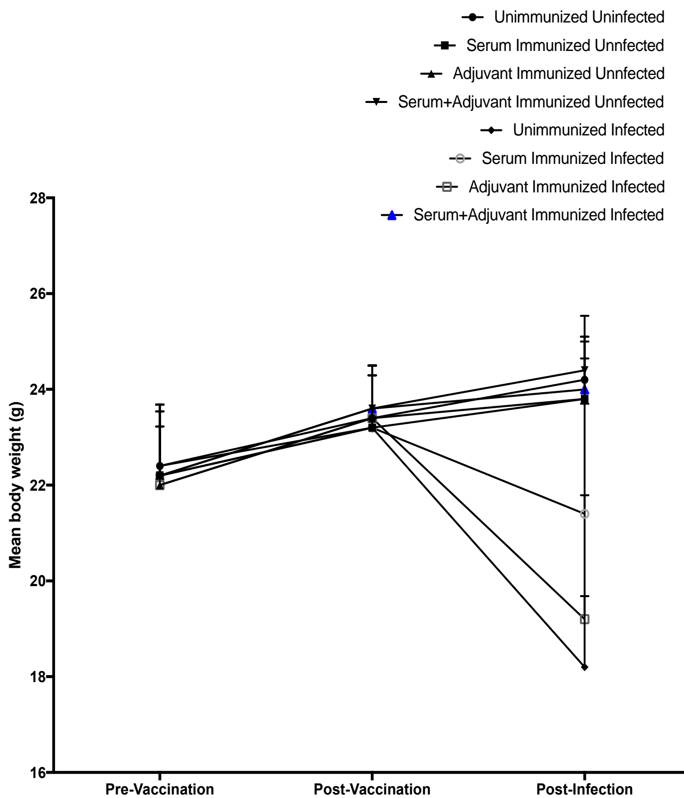

Figure 5: Mean body weight for the immunized and unimmunized infected and uninfected groups of experimental mice.

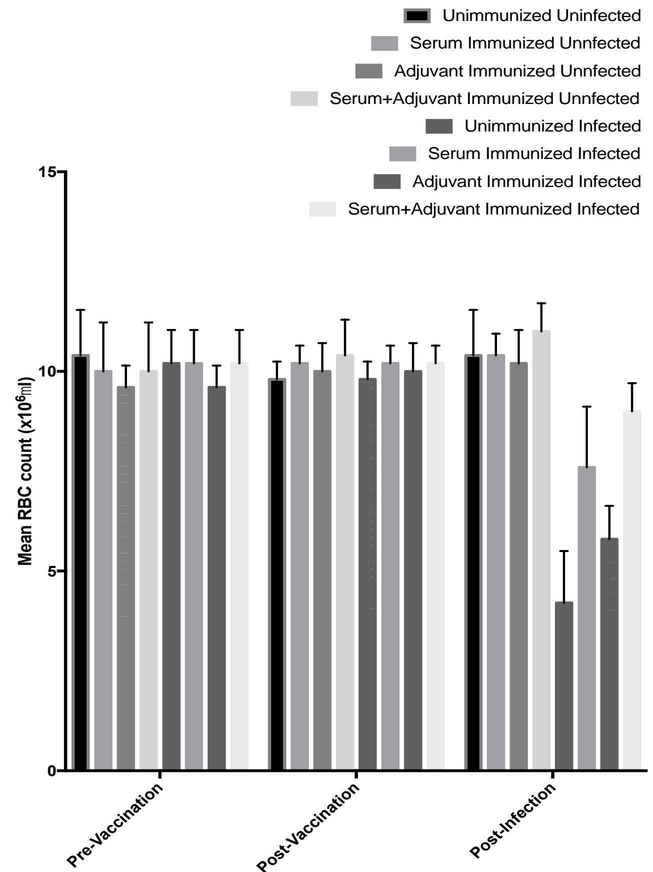

Figure 6: Mean RBC count for the immunized and unimmunized infected and uninfected groups of experimental mice.

In this study, we did not monitor antibodies that induce immunosuppression in the course of the infection. However, the differential WBC counts can give an idea with regard the immune responses observed in the groups of mice immunized with the antiserum.

We could speculate that the antiserum could possibly be acting synergistically and in concert with the adjuvant and this could be responsible for the elevated pre-patent period, parasitemia, weight, survival as well as the RBC and differential leucocyte counts observed in the serum-adjuvant immunized infected group but not in the serum-immunized infected group. Clearly, the adjuvant immunized infected groups did not differ significantly from the unimmunized infected group

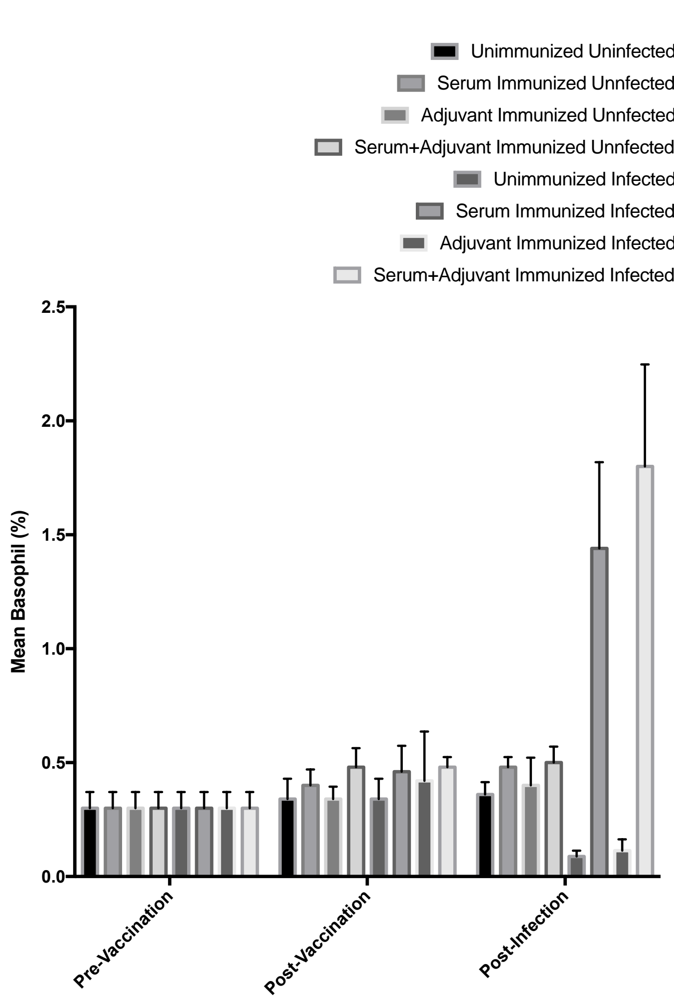

Figure 7: Mean Basophils for the immunized and unimmunized infected and uninfected groups of experimental mice.

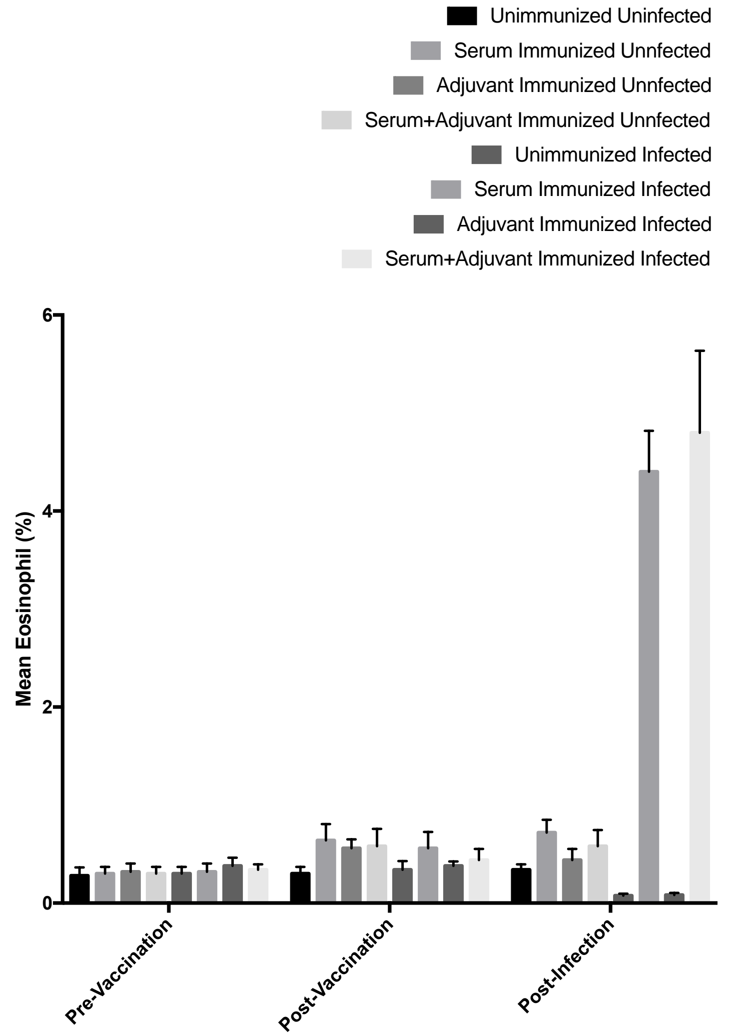

Figure 8: Mean Eosinophils for the immunized and unimmunized infected and uninfected groups of experimental mice.

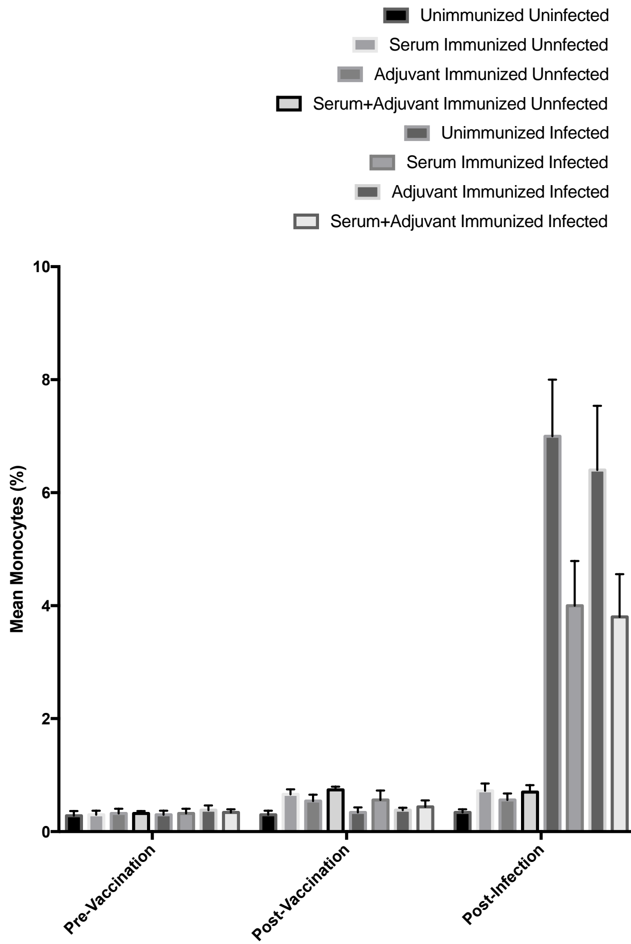

Figure 9: Mean Monocytes for the immunized and unimmunized infected and uninfected groups of experimental mice.

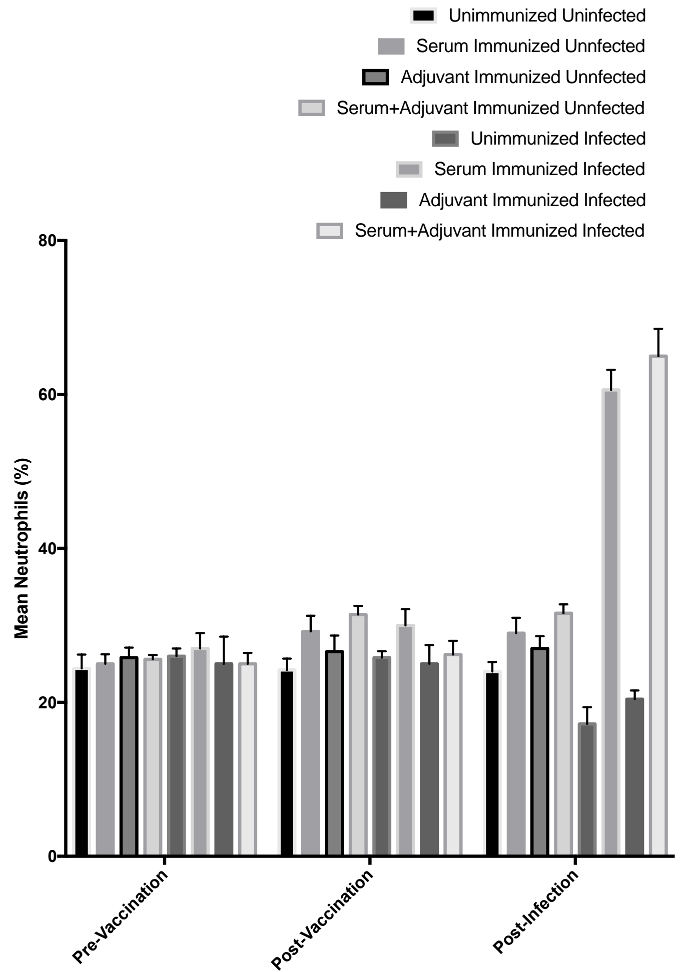

Figure 10: Mean Neutrophils for the immunized and unimmunized infected and uninfected groups of experimental mice.

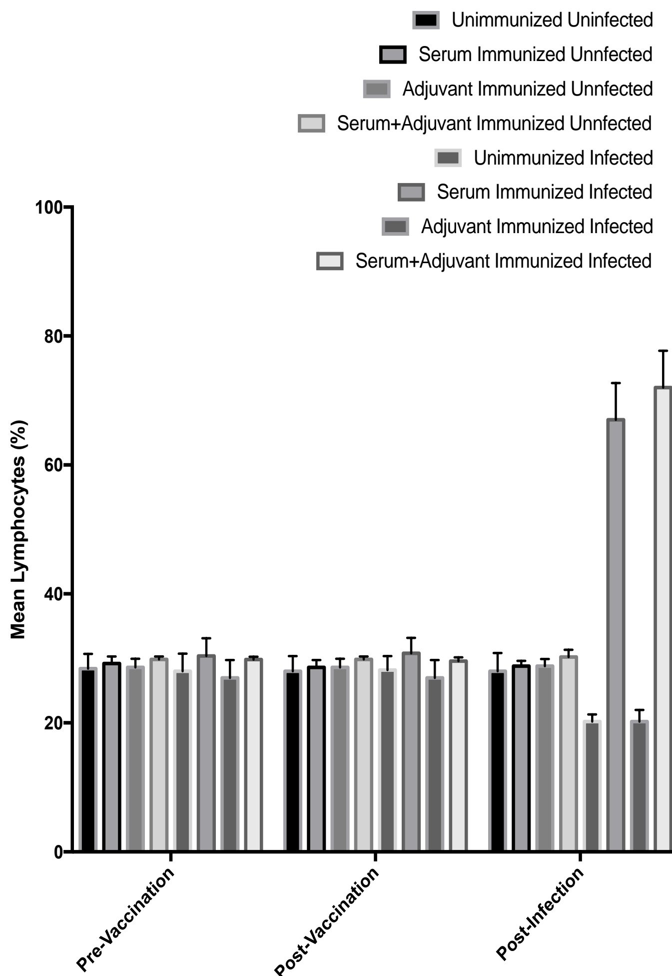

Figure 11: Mean Lymphocytes for the immunized and unimmunized infected and uninfected groups of experimental mice.

Antiserum is whole serum from blood of human or animals inoculated with an antigenic material or from those that recovered from a disease when they naturally developed certain antibodies against particular antigens that contains increased polyclonal IgG & IgM concentration to the specific immunogen including lots of other antibody specificities. Antisera are prepared to combat certain diseases. They are in particular used to provide passive immunity against diseases. The binding of antibodies to the antigens stimulates the immune system to carry out a stronger immune response against the antigen.

Adjuvants on the other hand are important tools required in slowly releasing antigens to stimulate the creation of antibodies (Petrovsky and Aguilar, 2004). Adjuvants may be added to a vaccine/immunogen to modify the immune system's response to the target antigen by boosting it such as to give a higher amount of antibodies and a longer-lasting protection but themselves do not provide immunity. Vegetable oils have been shown to be potential immunological adjuvants and have been shown to elicit humoral and cellular responses (Silva, 2004; Feijó, 2005; Sartor *et al.,* 2011). An outstanding leverage of vegetable oils is that they have minimal adverse side-effects for being biodegradable compounds (Ehhafona, 1996) The potentiality of the usage of peanut oil and other vegetable oils as adjuvant of the humoral and cellular immune response induced by *Leshmenia* antigens has been recently shown (Freitas *et al*., 2013). The peanut oil significantly increased the synthesis of anti-ovalbumin antibodies in the primary response, even though it did not favor cellular response. It was observed that the mice immunized with *Leshmenia amazonensis* antigens emulsified with peanut oil worsened the appearance of skin lesions and intensified lymph node parasite load suggesting stimulation of the Th2 immune response and down regulation of Th1 response (Freitas *et al*., 2013). This poor performance could not be unrelated to the variety of peanuts from which the oil was extracted and it could perform as a better adjuvant against other parasite antigens.

CONCLUSION

The data obtained in the present study showed that the serum and serum-adjuvant immunized infected groups survived for longer period of days than the unimmunized and adjuvant-

immunized infected groups. A delay in the on-set of parasitemia, display of parasitemia-wave as well as low parasitemia level accompanied by increased survivability of mice was observed in the serum and serum-adjuvant immunized infected groups. However, contrary observations were made in the unimmunized and adjuvant-immunized infected groups. Although some questions may be generated from the results presented in this work regarding, dosage of antiserum administered, time intervals between priming and boosting, route of administration and general mechanism of action of the antiserum since we did not monitor antibodies that induce immunosuppression in the course of the infection. Notwithstanding, the data generated shows some promising effectiveness of antiserum prophylaxis, which may offer some practical advantages when probably used in combination with a potential vaccine based regimen putting into consideration the bottle necks related to vaccine development strategies and the intrinsic mechanisms of immune memory development identified previously. We therefore recommended that some immune parameters be measured in order to understand the mechanism of immunoregulation in the course of the infection and also use of a more suitable host since the mouse-model may not be the most suitable representation of an optimal host-parasite context for the generation of relevant results, due to parasitaemia that may be a false representation of natural infections.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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