Asian Journal of Medicine and Health
1(6): 1-10, 2016; Article no.AJMAH.30065

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Immunostimulative Action of *Trypanosoma brucei* Phospholipase A_2 Gene Containing CpG Motifs Confers Homologous Protection against Infection in Experimental Mice

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AJN, HMI and IAU designed the study and wrote the protocol. Authors IYL and BY coordinated the laboratory studies. Author IYL led in producing the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJMAH/2016/30065
Editor(s):
(1) Triveni Krishnan, Division of Virology, National Institute of Cholera and Enteric Diseases, Kolkata, India.
Reviewers:
(1) Garba Muhammad Haruna, Federal University of Technology, Nigeria.
(2) Anonymous, National Research Centre on Equines, India.
(3) Samuel J. Black, University of Massachusetts, USA.
(4) De Trez Carl, Vrij Universiteit Brussel, Belghium.
(5) Michael Ezeani, Nnamdi Azikiwe University, Nigeria.
Complete Peer review History: http://www.sciencedomain.org/review-history/17121

Original Research Article

Received 14th October 2016
Accepted 29th November 2016
Published 3rd December 2016

ABSTRACT

Aim: Interest in phospholipase A_2 has continued to rise since its biotechnological potentials and involvement in the pathogenicity of *Trypanosoma* species were recognized, therefore this study was designed to investigate the vaccine potential of *Trypanosoma brucei* PLA_2 gene containing cytosine phosphate guanine (CpG) motifs against trypanosomiasis.
Place and Duration of Study: Department of Biotechnology, NVRI, Vom; Department of Parasitology, NITR, Vom and University of Jos, Nigeria between June 2011 and April, 2012.
Methodology: Bloodstream rat adapted strain of *T. brucei* was grown in rats and separated using

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DEAE cellulose chromatography. PCR amplification of phospholipase A_2 like gene from *Trypanosome brucei* cDNA synthesized by RT-PCR using parasites' RNA was done. Bioinformatics analyses of the gene sequence were done using Finch TV® programmes (GeoPiza) and Webgene programmes. Immunization of mice with DNA, immune sera and spleen extract were also carried out.

Results: A 1344 sequence obtained revealed CpG islands between positions 1 and 904. Immunization of mice with the PLA_2 DNA and in combination with partially purified parasite PLA_2 enzyme suppressed parasitemia as well as improved hematological indices and so enhanced survival of immunized mice post-infection. Passive immunization with immune sera and spleen extract showed suppressed parasitemia throughout the experimental period and delayed mortality to a greater extent compared to the DNA immunization.

Conclusion: The findings signify that DNA immunization confers protection on mice revealing the vaccine potential of PLA_2 like gene from *Trypanosoma brucei.*

Keywords: Trypanosoma brucei; PLA2; CpG Motifs.

1. INTRODUCTION

African trypanosomiasis is a tropical disease ranked among the most difficult Neglected Tropical Diseases [1]. This disease can cause 100% fatality when not detected and treated in time [2]. The infection increases by a minimum of 20,000 new cases a year, having over 50, 000,000 people at risk and killing a large number of cattle in Africa thereby causing further malnutrition [2,3]. *Trypanosoma brucei* causes nagana in cattle and serves as a model for studies of trials on *Trypanosomma* species [4]. The challenges being faced with *T. brucei* infection include situations where the parasite shows resistance to existing drugs [5,6]; most of the people affected or at risk live in the hardest to reach areas, not usually served by formal health systems [6] and the reality that the parasite has developed a mechanism to evade mammalian immune defense against the disease condition [7].

It is well documented that unmethylated cytosine-phosphate-guanosine (CpG) motifs exist in microbial DNA as a normal phenomenon [8,9]. These motifs are recognized and treated as a danger signal by the innate immune system [10,11] as well as have been demonstrated to have immunostimulatory activity in vertebrates [12,13]. However, protective effect of gene from protozoan parasites against parasite infection has not been demonstrated. Therefore, the study was designed to test the efficacy of PLA_2 gene from *T. brucei* as a vaccine candidate against the parasite infection in experimental mice and to assay other parameters such as packed cell volume and animal mortality. *T. brucei* phospholipase A_2 is a membrane associated hydrolytic enzyme belonging to a superfamily known to catalyse the hydrolysis of the sn-2 ester bond of phospholipids realing free fatty acids and lyso-phospholipid [14,15,16]. This enzyme has been implicated in anemia during trypanosomiasis due to its haemolytic tendency [17]. Therefore, an attempt to arresting its activity may provide a protection against *T. brucei* infection as well as check the severity of anaemia in trypanosomiasis.

2. MATERIALS AND METHODS

2.1 Experimental Animals and Experimental Design

This study was carried out in accordance with guidelines for the Care and Use of Laboratory Animals in University of Jos, Nigeria. Albino rats were used for propagation of *Trypanosoma brucei* while albino mice were used for immunization tests. *Trypanosoma brucei* infected blood was supplied by the Parasitology Department, Nigerian Institute of Trypanosomiasis Research (NITR), Vom, Nigeria.

The Immunization studies using the PLA_2 DNA as potential vaccine against trypanosomes was done according to a method reported earlier [18]. Twelve groups of 6 mice each were conditioned for the experiment. The immunization regimen was Prime-Double boost immunization (0-7-14) while immunogen concentrations were 20 µg DNA/dose and 20 µg PLA_2 (protein)/dose). Vaccination was done intraperitoneally and infected on day 21 with about 50 µl of 1 $x10^3$ cells/ml and monitored via tail snip. Pre-immune bleeding was done on day 0. The process was carried as in Table 1.

Table 1. Details of experimental groups and treatments

Group	Experimental design
T1	Primed and double boosted on days 7 and 14 with PLA_2DNA then challenged with *T. brucei* on day 21; parasitemia, PCV and mortality were monitored
T2	Primed with DNA and double boosted with PLA_2 protein then infected on day 21; parasitemia, PCV and mortality were monitored
C1	Not immunized but infected on day 21; parasitemia, PCV and mortality were monitored
C2	Not immunized and not infected.
T1a	Primed with PLA_2 DNA and boosted once with PLA_2 DNA, then sacrificed on day 14 (First boost); serum prepared for humoral response studies.
T1b	Primed with PLA_2 DNA and double boosted with PLA_2 DNA, then sacrificed on day 21(Second boost); serum and spleen extract prepared for humoral response and passive immunization studies.
T2a	Primed with PLA_2 DNA and boosted once with PLA_2 protein, then sacrificed on day 14 (First boost); serum prepared for humoral response studies.
T2b	Primed with PLA_2 DNA and double boosted with PLA_2 protein, then sacrificed on day 21(Second boost); serum and spleen extract prepared for humoral response and passive immunization studies.
T1 antiserum	Immunized with 0.2 ml of antiserum prepared from T1b mice; parasitemia, humoral response and PCV were monitored
T1 spleen extract	Immunized with 0.2 ml of spleen extract prepared from T1b mice; parasitemia, humoral response and PCV were monitored
T2 antiserum	Immunized with 0.2 ml of antiserum prepared from T2b mice; parasitemia, humoral response and PCV were monitored
T2 spleen extract	Immunized with 0.2 ml of spleen extract prepared from T2b mice; parasitemia, humoral response and PCV were monitored

2.2 RNA Isolation and PCR Amplification of Phospholipase A_2 like Gene

Parasites were purified on DEAE-cellulose (pre-swollen whatman DE-52-Pharmacia Fine Chemicals) base on a describd method [19]. RNA was extracted from 100 µl of isolated *T. brucei* suspended in PSG buffer pH 8.0 using ZR Viral RNA Kit (Zymo Research) according to the Manufacturer's instructions. The RT-PCR kit (Thermo Fisher Scientific) was used for cDNA synthesis according to manufacturer's instruction. The Mix in 50 µl was as follows: H_2O (14.0 µl), 5 x RT buffer (04.0 µl), $MgCl_2$ (08.0 µl), dNTP mix (04.0 µl), Oligo dT (04.0 µl), RNase inhibitor (2.0 µl), Reverse transcriptase (04.0 µl), RNA (10.0 µl). The mix was incubated in Block Heater (Stuart Scientific®) at 37℃ for 1hour after which it was inactivated at 85℃ for 5 minutes.

The phospholipase A_2 like gene was amplified from the cDNA by Polymerase Chain Reaction (PCR) with primers designed in Inqaba Biotech Industry, Pretoria, South Africa based on the gene sequence of PLA_2 like gene in the Gene Bank Data Base (Tb 09.211.3650, Phospholipase A_2-like protein, putative, *T. brucei*, chr 9). The primers used were: Sense primer 5'-ATGGTAACGT GGGCGCTGAA GTAT- 3' and Anti-sense primer 5'-CTAACACGTTGAACACA CTTCGGTA-3'. The thermal cycling was carried out according to a procedure earlier described [20] and the results documented using Gel Documentation System (Synegene®).

2.3 PLA_2 Preparation and Assay

The preparation of soluble PLA_2 proteins was done according to a method reported earlier [21]. The crude enzyme extract or soluble protein obtained was purified using Chromatography on DEAE- Cellulose [22] and Gel Filtration on Sephadex G-50. Recovered fractions showing PLA_2 activity were used. The PLA_2 activity was determined by the hydrolysis of L-α-phosphatidyl choline (L-α-lecithin) (Sigma); 25 µl of this substrate was incubated with 10 µl of fraction (in 50 mM Tris/HCl buffer, pH 8.0) and 10 µl of distilled water. The mixture was incubated for 10 min, then immersed in boiling water for 2 min to stop the reaction. The liberated fatty acids were titrated against 20 mM NaOH, using phenolphthalein as indicator. Protein concentrations were determined using the Bradford method with Bovine Serum Albumin in 2 µg, 4 µg, 6 µg, 8 µg and 10 µg/ ml concentrations as standard and read against reference at 595 nm in an Ultrospec 3000 spectrophotometer.

2.4 Measurement of Antibody Responses

Serum samples from mice immunized with PLA_2 DNA were separately assayed for anti PLA_2 gene antibodies by enzyme-linked immunosorbent assay. Polyvinyl chloride plates (96-well) were coated overnight at 4℃ with 100 µl of *T. brucei* lysates. Plates were blocked for 2 hours at 37℃ with 200 µl/well of 1% non-fat dry milk in PBS and washed with PBS-0.05%. The plates were incubated for 2hours with test sera (100 µl/well). The mixture was incubated at room temperature for 30 minutes with 100 µl of horseradish peroxidase labeled anti-mouse immunoglobulin. The colour was developed with 100 µl of ABTS [22' azinobis (3-ethylbenzthiazolinesulfonic acid)] /well and optical density read at 405nm micro plate reader.

2.5 Packed Cell Volume (PCV) Estimation

Blood was collected into capillary tubes with anticoagulant directly from tail snip to about 75% of its length before the ends were sealed with plastacin. The capillary tubes and their contents were centrifuged for 5 min in a microchaematocrit centrifuge. The spun tubes were placed in the Microhaematocrit reader and the PCV read in percentage.

2.6 Bioinformatics Analyses

Finch TV® programmes (GeoPiza), and Webgene programmes were used to analyse the sequence.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 CpG island mapping

The results revealed that the PLA_2 sequence had CpG islands between positions one and nine hundred and four. The CpG islands displayed the following properties: the CG content was 50%; CpG content was 6%; the ratio of observed-to-expected number of CpG dinucleotides was 0.99 and sequence length of 1344bp (Fig. 1).

3.1.2 Effect of DNA immunization on parasitaemia and animal survival

The ability of the PLA_2 DNA from bloodstream form *T.brucei* to homogeneously protect mice against *T. brucei* infection in T1 group mice evaluated showed that immunization with PLA_2 DNA suppressed parasitaemia between days 9 and 11 post infection. The immunized mice had a decrease in parasitaemia to the lowest level of about 4 per field (3.6×10^6 cells/ml) 9 days post infection (Fig. 2).

The results from the T2 group showed that the immunization elicited suppressed parasitaemia between the 5^{th} and the 12^{th} day post infection. Parasitemia decreased to as low as one parasite per field particularly between days 7 and 9 (Fig. 2) as detected by direct microscopic examination. This is a more effective result than the T1 group.

The immunization of the animals in both T1 and T2 groups revealed some effects on their survival. Two (33.33%) of the control groups died by day 7 while the remaining 66.66% died by day 8 post infection. In the immunized groups some of the animals lived for a longer period up to day 15 (T1 group) and day 16 (T2 group) post infection (Fig. 3).

3.1.3 Effect on packed cell volume

On day zero both the control and test group animals had high PCV levels. However, on day 7 the PCV levels of all the groups dropped. By day 10, only the T1and T2 group members were alive having values of (41.7±5.9) and (48.1±1.5) respectively (Table 2). These tend to show a significant increase ($p<0.05$) compared to the control values on day 7.

Table 2. Effect of PLA_2 naked DNA immunization on PCV (%)

	Day 0	Day7	Day 10
Control	47±3.7	34.9±0.9	
T1	47.3±5.8	$38.1±0.9^a$	$41.7±2.2^b$
T2	49±6.5	$40.2±1.1^a$	$48.1±1.5^b$

Control group was not immunized but infected; T1 group was primed and double boosted with PLA_2 DNA; T2 group was primed with PLA_2 DNA and double boosted with PLA_2 protein; [a] indicates values not significantly different from control; [b] indicates values significantly different from the control at $p<0.05$

3.1.4 Effect of passive immunization on parasitaemia and survival

The passive immunization studies involving the PLA_2 DNA anti-sera and spleen extract from immunized mice revealed similar parasitaemia pattern in the control, the T1 anti serum and T2 anti-serum groups within the first 7 days post infection. There was a rise in parasitaemia to day

8 when the control animals all died. On the other hand, in the T1 and T2 anti-sera groups there was a drop in the level of parasites from day 7 to day 14 post infection. The parasitaemia then rose until day 15 (T1 anti-serum) and day 16 (T2 anti-serum) when the animals had all died. The T1 and T2 spleen extract groups exhibited a suppressed parasite level, compared to the control group, throughout the experimental period.

ID	Query
	Results of CpG islands mapping
URL:	www.itba.mi.cnr.it/webgene
Key	Location/Qualifiers
	CpG_island 1..904
	CpG island: %C+G= 50; %CpG= 6, Obs./Exp.= 0.99
SQ	1344 BP; 311 A; 307 C; 341 G; 385 T; 0 other;

Fig. 1. CpG islands mapping of PLA_2 like gene sequence from *T. brucei*

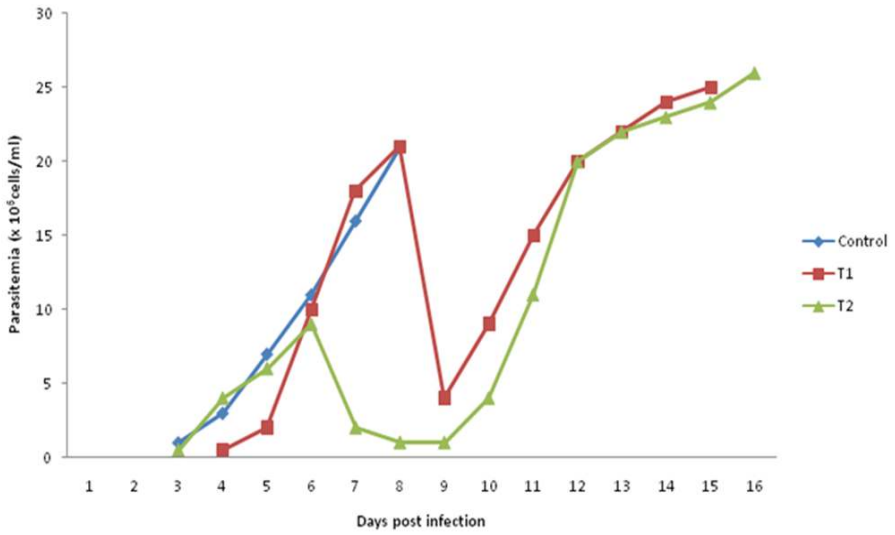

Fig. 2. Parasitemia in immunized mice

Control group was not immunized but infected; T1 was primed and double boosted with PLA_2 DNA; T2 group was primed with PLA_2 DNA and double boosted with PLA_2 protein

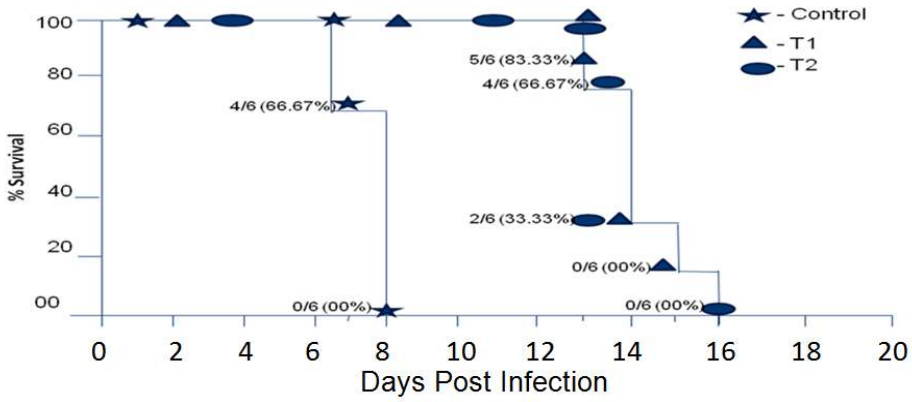

Fig. 3. Survival in immunized and infected mice

Control group was not immunized but infected; T1 group was primed and double boosted with PLA_2 DNA; T2 group was primed with PLA_2 DNA and double boosted with PLA_2 protein

3.1.5 Effect of passive immunization on PCV

Packed Cell Volume values read revealed the different level of anaemia which is one of the clinical signs of trypanosomiasis. The PCV values were not significantly ($p<0.05$) different from the control values on day 7 post infection (Table 3). On day 12 PCV values became significantly different ($p<0.05$) compared to the control values on day 7.

3.1.6 Effect of the immunization on humoral response in mice

Levels of Immunoglobulin G secreted in response to the immunogens used in the immunizations were detected in the experimental animals and at the different stages of immunization. In both test groups (T1 and T2), the prime immunization with PLA_2 DNA gave a significant ($p< 0.05$) level of IgG compared to the control. Also, the first (on day 7) and second (on day 14) boosts with DNA as well as the first and second boost with partially purified PLA_2 (T2 group) gave a significantly higher IgG level ($p <0.05$) compared to the control (Fig. 5).

3.2 Discussion

The lowering of parasitaemia in the test animals, when compared to the controls, demonstrated the protective effect of *T. brucei* PLA_2 DNA, PLA_2 protein, the immune sera and spleen extract. Trypanotolerance was ruled out since all the control (infected without immunization) groups did not show such reduced parasitaemia at any stage of the experiment. Equally, variable surface glycoprotein (VSG) coat molecules are highly immunogenic and provoke both B and T cells response that are directed at eliminating parasites from blood and other tissues [23], but the anti-trypanosome effect was not observed in the control groups suggesting that PLA_2 DNA anti-trypanosome effect was exhibited.

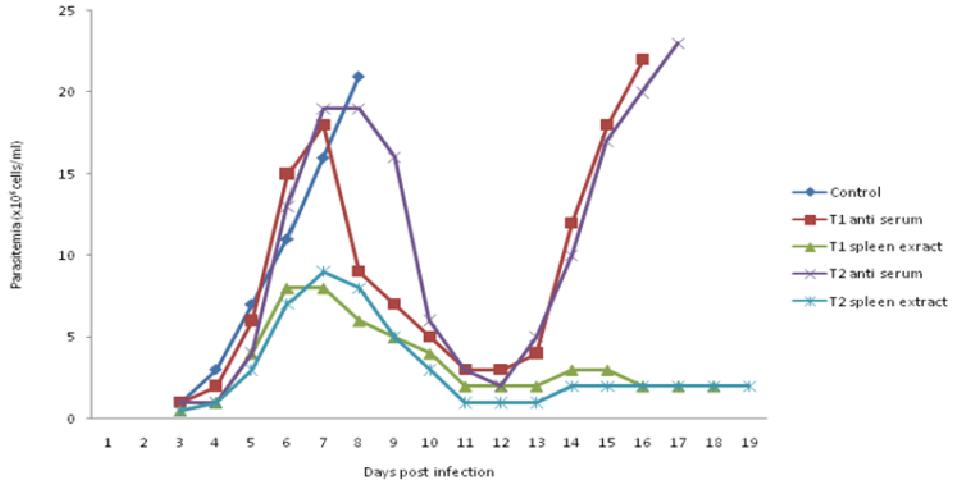

Fig. 4. Parasitemia levels in passively immunized mice

Control group was not immunized but infected; T1 group was primed and double boosted with PLA_2 DNA; T2 group was primed with PLA_2 DNA and double boosted with PLA_2 protein

Table 3. Effect of anti-serum and spleen extract on PCV (%)

	Day 0	Day7	Day 12
Control	48.0±1.2	33.1±0.8	
T1 antiserum	47.1±2.0	34.4±1.7 [b]	39.8±3.6 [b]
T1 spleen extract	48.6±3.0	38.2±2.4 [b]	41.2±3.1 [b]
T2 antiserum	46.7±2.1	34.6±2.8 [b]	40.2±2.1 [b]
T2 spleen extract	47.8±1.5	37.4±4.0 [b]	42.4±3.2 [b]

Control group was not immunized but infected; T1 was primed and double boosted with PLA_2 DNA; T2 group was primed with PLA_2 DNA and double boosted with PLA_2 protein; a indicates values not significantly different from the control; [b] indicates values significantly different from the control

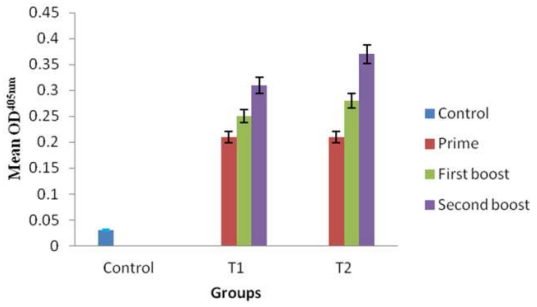

Fig. 5. Humoral response in immunized mice
Control group was not immunized but infected; T1 group was primed and double boosted with PLA_2 DNA; T2 group was primed with PLA_2 DNA and double boosted with PLA_2 protein

The immunogens were immunostimulative as shown by the levels of IgG after the prime and each boost. The higher IgG levels in the mice (T2) primed with PLA_2 gene and double boosted with the protein than in the (T1) double boosted with DNA tend to explain the basis for the former's higher effect on the parasites *In vivo.* The immunostimulative action of T2 immunogens from *T. brucei* is in accord with reports on this quality in PLA_2 protein obtained from other organisms [24,25,26]. On the other hand, the effect of the PLA_2 DNA on the immune system in this work agrees with reports in literature that naked DNA has immunogenic effects in mice [27,28]. The capacity of PLA_2 DNA to produce this result may be due to its CpG motif. This is most likely because of the several reports on the potentials of DNA carrying this motif particularly if it is unmethylated. Literature reports hold that DNA containing an unmethylated CpG motif has a potent immunostimulatory effect on the vertebrate immune system [29,30,7,31,32,33,34] and that most microbial CpGs are unmethylated and their DNA in gene therapy induces direct immune stimulation through activating the host defense mechanism triggering rapid activation of B cells, monocytes, macrophages, dendritic cells, and natural killer cells, along with the release of pro-inflammatory cytokines [3,11]. Therefore, it becomes convincing to link the trypanocidal activity of PLA_2 DNA immunization to the presence of the CpG motif when considered against a report that the strong and long-lasting antigen-specific humoral (antibodies) and cell-mediated (T help, other cytokine functions and cytotoxic T cells) immune responses induced by DNA vaccines appear to be due to the sustained *In vivo* expression of antigen, efficient antigen presentation and the presence of stimulatory CpG motifs [35]. This is further buttressed by the report that CpG motif stimulates immunity against tuberculosis infection [36].

The passive immunization with spleen extract gave suppressed parasitaemia throughout the period of the study showing a good level of efficacy against *T. brucei* infection. Considering the role of spleen as the major site of immune responses to blood borne antigens, it is conceivable that the spleen extract had high concentration of PLA_2 DNA antibodies which suppressed parasitaemia within the period of the study. Passive immunity has been reported to be a useful way of conferring resistance in relatively fast way because the spleen cells from DNA immunized mice had high levels of IFN- γ and II-12 cytokines which was linked to the protection of mice against *T. gondii* infection [37]. This may account for the suppressed parasitaemia and higher percentage survival observed in the experimental animals immunized with the spleen extract compared to the ant-serum immunized mice and the control group.

4. CONCLUSION

It can be concluded that PLA_2 DNA and passive immunization with anti-serum to PLA_2 DNA were only able to suppress parasitaemia for a short period and cannot be said to have conferred significant protection against the parasite infection in mice. On the other hand, passive immunization with the spleen extract suppressed parasitaemia for a longer period of time. The study has therefore implicated PLA_2 DNA in immune stimulation leading to IgG production against *T. brucei* infection.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Animal experiments were carried out in accordance with the instructions for the care and use provided by the University of Jos, Nigeria where part of the research was carried out. The experiments were examined and approved by the University of Jos animal use ethics committee.

ACKNOWLEDGEMENTS

We acknowledge the Tertiary Education Trust Fund (TETFUND), Abuja, Nigeria for supporting

this work with grant. We appreciate the technical assistance of the staff of the Molecular Biology Laboratory, NVRI, Vom, Nigeria as well as the kindness of the staff of the Parasitology Department, NITR, Vom, Nigeria for supplying the parasites.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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